Defining Toxicological Endpoints of Aphis Gossypii Feeding

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DEFINING TOXICOLOGICAL ENDPOINTS OF *APHIS GOSYPHII* FEEDING

A Thesis
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree
Master of Science

in
The Department of Entomology

by
Flinn O’Hara
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Abstract

The cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), is a destructive agricultural pest, due to photosynthate removal and plant virus transmission. This project consisted of two main goals. First, we employed toxicity bioassays and electrical penetration graph (EPG) methodology to quantify changes to feeding behavior and toxicity of *A. gossypii* after exposure to commercialized aphicides. Commercialized aphicides containing flupyradifurone, sulfoxaflor, thiamethoxam, thiamethoxam + lambda cyhalothrin, and bifenthrin induced > 90% aphid mortality within 4 hours of exposure. This was supported by our EPG results that showed a significant reduction in the proportion of aphids that continued to probe on cotton 4 hr after exposure to flonicamid, thiamethoxam, flupyradifurone, bifenthrin, and thiamethoxam premixed with lambda cyhalothrin. Furthermore, flupyradifurone induced an LT$_{50}$ of 8.9 min after exposure. Lastly, phloem feeding was significantly reduced for flupyradifurone, flonicamid, thiamethoxam, sulfoxaflor, and thiamethoxam. In the second goal, we aimed to test the antifeedant properties of small-molecule inhibitors of inward rectifier potassium (Kir) channels expressed in the aphid salivary glands. Two Kir channel inhibitors, VU041 and VU730, reduced the secretory activity of the aphid salivary gland by 3.3-fold and importantly, foliar applications of VU041 and VU730 significantly increased the time to first probe, total probe duration, and nearly eliminated ingestion of phloem. Although promising, foliar applications of chemicals have significant pitfalls including non-target toxicity and increased costs of application. Thus, we tested the capability of a novel natural product based solubilizer to increase systemic movement of VU041 and VU730, which are highly lipophilic molecules. To study systemic movement throughout the plant and across the leaf surface, the adaxial side of a single cotton plant leaf was treated with a Kir inhibitor solubilized into the water-soluble concentrate. Upper leaves were infested with aphids 60-72 hours after chemical treatment and changes to
feeding behavior were quantified through EPG methodology. Trans-laminar and translocation of Kir modulators was confirmed as we observed a significant reduction of aphids able to reach phloem. These data further support hemipteran Kir channels as a target to prevent feeding and plant virus transmission through novel delivery mechanisms that enable plant systemic movement.
Chapter 1. Literature Review

1.1. Cotton Aphid (*Aphis Gossypii*) Pest Status

Aphids are polyphagous hemipterans that are one of the most damaging group of pests in agriculture and contributes to economic losses exceeding $1 billion annually. Of aphids, the polyphagous cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), is one of the most destructive agricultural pests. *A. gossypii* has a world-wide distribution with a host range of over 92 plant families, which includes causing potential damage to a number of economically important crops such as: cotton (*Gossypium hirsutum* L.), cucurbits (Cucurbitaceae), aubergine (*Solanum melongena* L.), citrus (Rutaceae), watermelon (*Citrullus lanatus* Thunb.), okra (*Abelmoschus esculentus* L.), and potatoes (*Solanum tuberosum* L.).

These economic losses can stem from direct feeding damage due to the ingestion of plant phloem sap, and through indirect damage via honeydew excretion and virus transmission, respectively. Photosynthate removal reduces nutritional resources that can have adverse physiological effects on the plant such as wilting, stunted growth in younger plants, and potential plant death. For example, studies carried out on cotton in the United States in 1991 observed that 10 million of 13 million acres harvested were infested with aphids, which resulted in 360,000 cotton bales being lost due to the effects of *A. gossypii* direct feeding damage. Yield losses were at their highest in Texas, where a 6% yield loss was observed. In terms of indirect damage, the excretion of sugar-rich honeydew from *A. gossypii* reduces fruit marketability and can contaminate cotton lint, which leads to a decrease in cotton quality and economic value. Additionally, the presence of sugar-rich honeydew can help protect *A. gossypii* from natural enemies by potentially attracting bees, wasps and ants to the plant. However it is the ability of aphids to transmit viruses that has the largest impact on agriculture, with *A. gossypii* being able to transmit over 50 viruses.
1.2. Cotton Aphids as a Disease Vector

Plant virus acquisition, transmission and retention occurs via different modes and these modes are dependent upon virus location within the insect and plant tissues. Plant viruses also vary in their latent periods, which can be defined as the duration between a vector acquiring and subsequently being able to transmit this virus. Nonpersistent plant viruses tend to be stylet-borne with no latent period, where they can be acquired and transmitted within seconds to minutes of aphid mouthparts penetrating the leaf epidermis. The majority of nonpersistent viruses are transmitted by aphids and are lost after molting. Semipersistent viruses are located in either the stylet or foregut and are transmitted over minutes to hours of probing. Semipersistent viruses also have no latent period and are lost after aphid molting. Plant viruses that are persistent circulative or persistent propagative are typically located within the plant vascular bundles that are acquired and transmitted over hours to days due to the latent period of the viruses. Persistent circulative viruses can be retained in the aphid for days to weeks, whilst persistent propagative viruses can be retained for the entire lifespan of the insect due to virus replication within the vector. An example of persistent circulative viruses are luteoviruses, which are restricted to phloem sieve elements, companion cells and phloem parenchyma cells. These persistent circulative viruses are acquired during phloem sap ingestion of an infected plant and passes across the hindgut epithelium into the haemolymph. Virus transmission occurs when the virus is transferred to the accessory salivary gland, where it can be injected with the saliva into a healthy plant sieve element. An emphasis will be placed on changes to feeding behavior related to persistent virus transmission in this study, but it is important to consider that the mode of virus transmission can directly affects management approaches.
1.3. Cotton Aphid Chemical Control Methods

Broad applications of nervous system targeting synthetic insecticides are still the primary control methods for reducing aphid populations. This is supported by the list of approved modes of action (MoA) for aphid control by the Insecticide Resistance Action Committee (IRAC), which is listed in Table 1.1. The effective control of plant virus vectors is critical for minimizing the spread of plant viruses in crops, which has occurred to varying degrees of success. For instance, previous studies have demonstrated that chemical compounds can disrupt but not eliminate the transmission of phloem-restricted viruses, by disrupting aphid ability to reach and continuously feed from phloem. A recent paper even demonstrated that flonicamid treated plants were able to significantly reduce the acquisition rate and subsequent inoculation of the phloem-restricted luteovirus turnip yellows virus (TuYV) for Myzus persicae Sulzer (Homoptera: Aphididae). This paper provides evidence that synthetic insecticides can play an important role in minimizing the spread of plant viruses as well as the effective control of vector populations. Failure of insecticides to prevent phloem-restricted virus acquisition and transmission can occur if the aphid mouthparts reach the sieve elements and continuously feed from them prior to insect death. One additional consideration for the control of persistent viruses is that persistent viruses are not only transmitted by aphids that can colonize plants, but by those that have a nearby colony and have the potential to vector persistently transmitted viruses. Therefore, a control product to prevent persistent virus transmission would need to control aphid populations, as well as disrupt sieve-element salivation and phloem ingestion of individual aphids.
It is important to consider that the majority of cases demonstrating the successful control of virus transmission through insecticide use were for semipersistent and persistent transmitted viruses \(^{31}\) and not nonpersistent viruses. Our previous studies have shown that aphid probing occurs between 4-6 minutes of landing on the plant \(^{36}\), with nonpersistent viruses occurring within minutes to seconds of probing \(^{24}\). Insecticides must be able to kill the vector

<table>
<thead>
<tr>
<th><strong>IRAC MoA Number</strong></th>
<th><strong>Primary Mode of Action</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbamates (1A)</td>
<td>Acetylcholinesterase (AChE) inhibitors</td>
</tr>
<tr>
<td>Organophosphates (1B)</td>
<td>Acetylcholinesterase (AChE) inhibitors</td>
</tr>
<tr>
<td>Pyrethroids – Pyrethrins (3A)</td>
<td>Sodium channel modulators – Nerve action</td>
</tr>
<tr>
<td>Neonicotinoids (4A)</td>
<td>Nicotinic acetylcholine receptor (nAChR) competitive modulators – Nerve Action</td>
</tr>
<tr>
<td>Sulfoximines (4C)</td>
<td>Nicotinic acetylcholine receptor (nAChR) competitive modulators – Nerve Action</td>
</tr>
<tr>
<td>Butenolides (4D)</td>
<td>Nicotinic acetylcholine receptor (nAChR) competitive modulators – Nerve Action</td>
</tr>
<tr>
<td>Pyridine azomethine derivatives (9B)</td>
<td>Chordotonal organ TRPV channel modulators – Nerve action</td>
</tr>
<tr>
<td>Diafenthiuron (12A)</td>
<td>Inhibitors of mitochondrial ATP synthase – Energy metabolism</td>
</tr>
<tr>
<td>Tetronic and Tetramic acid derivatives (23)</td>
<td>Inhibitors of acetyl CoA carboxylase – Lipid synthesis, growth regulation</td>
</tr>
<tr>
<td>Flonicamid (29)</td>
<td>Chordotonal organ modulators – undefined target site – Nerve action</td>
</tr>
</tbody>
</table>

Table 1.1. List of insecticides for aphid control by IRAC (Insecticide Resistance Action Committee) MoA number, and primary mode of action. Adapted from \(^{30}\).
or alter vector feeding behaviour within this time to stop or limit acquisition and inoculation of nonpersistent viruses to plants. However, a recent study found that current commercialized aphicides were not able reduce nonpersistent virus transmission\(^\text{37}\). It is hypothesized that the vector does not interact with the plant long enough to receive a lethal dose of insecticide prior to vector mouthparts penetrating the leaf epidermis. In fact, some products such as pyrethroids have been shown to induce vector agitation and increase transmission of nonpersistent viruses due to increased plant-to-plant visitation\(^\text{28, 29}\). Thus, understanding how insecticides alter aphid feeding behaviour is important for building a pest management plan to reduce virus transmission.

As represented by Table 1.1., applications of synthetic insecticides targeting the nervous system remain the primary method for controlling aphids in agricultural settings, yet the development of insecticide resistance has reduced the efficacy of control\(^\text{30, 38, 39}\). The problem of resistance is amplified for aphids as the rate of insecticide resistance evolution is increased due to parthenogenesis, a form of asexual reproduction which could allow for dozens of generations of resistant offspring within a single growing period\(^\text{39}\). Control failures are common with \(A.\ gossypii\) as they are highly resistant to the majority of approved chemical classes through increased metabolic detoxification\(^\text{40}\) and target site mechanisms\(^\text{40, 41}\). Current research has already shown evidence of \(A.\ gossypii\) resistance to: pyrethroids\(^\text{39}\); lipid biosynthesis inhibitors\(^\text{42}\); and neonicotinoids\(^\text{43, 44}\); carbamates\(^\text{45}\); and organophosphates\(^\text{45}\). Resistance to the majority of approved insecticidal classes has underscored the importance of developing novel mode and mechanism insecticides to compliment current hemipteran control measures\(^\text{46}\).
1.4. Understanding Aphid Feeding Biology

It is difficult to quantify plant damage of piercing sucking insects as the cellular punctures and feeding behaviors relevant to virus transmission occur within the plant and cannot be directly observed without disrupting the insect. However, these probing behaviors can be observed using the electrical penetration graph (EPG) technique, which was first carried out by Mclean and Kinsey in 1964.47 Gold wire was fixed onto the dorsum of the aphid with silver glue before the aphid is placed onto a plant with a conductive grid being hung over the plant tissue.47 A complete circuit is formed when aphid stylets penetrate the leaf epidermis, referred to as a probe.47 A waveform is produced by the resistance created from aphid salivation, mouth part movements, and the variation of membrane potentials between different plant tissues.48

The EPG technique has been improved and refined into AC and DC systems, which produce different waveforms depending on aphid feeding behavior that can be observed and quantified.48 A combination of histological studies, artificial diet, and direct observation of aphid stylets have helped elucidate and categorize seven different waveform patterns through EPG, which are labelled from A to G and pd.25, 48, 50, 51 An example of each of these waveforms along with a summary of the feeding behavior and its location within the plant are described in Figure 1.1. EPG recording can additionally be utilized to understand how aphid feeding behaviors and the duration of those behaviors vary under different experimental conditions. These studies can elucidate feeding behavior differences between: different species of host plants, different plant varieties, different aphid species, insecticide exposure, and starved aphids compared to non-starved aphids.54 The EPG technique has been adapted to a variety of different piercing-sucking insects including: whiteflies, mosquitoes, varroa mites, ticks, and planthoppers.
Fig. 1.1. Electrical penetration graph waveforms. Waveforms A refers to stylet contact with the leaf epidermis. Waveform B is salivation in the epidermis and mesophyll, forming the salivary sheath \(^{48}\). Waveform C is related to intercellular apoplastic stylet pathway in all plant tissues. Waveform F indicates derailed stylet mechanics in all tissues. Waveform G indicates the active intake of xylem sap \(^{48}\). Potential drops (pd) are intracellular punctures that can occur within any living cell when aphids take sap samples \(^{59},^{60}\). Aphid mouthparts reaching sieve elements (waveform E) can be broken down into two distinct subphases, which are sieve-element secretion of watery saliva (E1) and passive phloem ingestion (E2) \(^{25}\). Pattern E2 is always preceded by E1, which would indicate that the secretion of saliva in sieve elements (E1) is required for passive phloem ingestion (E2) \(^{25}\).

It also possible to use the EPG technique to further understand virus transmission and acquisition by interpreting the different waveforms. Aphid host selection partly occurs due to gustatory organs present in the aphid foregut, which can only function by probing the plant and ingesting sap and sampling cell contents \(^{59},^{60}\). This sampling of cell contents can be visualized as a distinct potential drop (pd) on EPG recordings (Fig. 1.1.), due to the voltage difference between cells and the extracellular space \(^{48}\). These potential drops are broken down into three subphases (II-1, II-2, and II-3) that are relevant to nonpersistent virus
acquisition and transmission. Nonpersistent virus transmission is related to the duration of aphid salivation (phase II-1) into plant cells. On the other hand, nonpersistent virus acquisition occurs through the ingestion of cell contents (phase II-3) and can be correlated with the number of archlets present as well as the duration of this subphase. An increase in the duration of phase II-1 and II-3 are associated with increases in nonpersistent virus transmission and acquisition, respectively.

Furthermore, the EPG technique allows us to understand how phloem-restricted viruses can be acquired and transmitted. Aphid sieve element phase consists of two distinct subphases, which are sieve-element secretion of watery saliva (E1) and passive phloem ingestion (E2). Pattern E2 is always preceded by E1, meaning that the secretion of aphid watery saliva (E1) is required for passive phloem ingestion (E2). The duration of E1 is important for persistent virus transmission because aphids inoculate the virus during the sieve element salivation phase (E1). On the other hand persistent virus acquisition occurs during prolonged phloem sap ingestion (E2). Therefore, understanding how commercialized aphicides could alter the durations of E1 and E2 waveforms can be related to persistent virus transmission.

There are multiple studies providing evidence that the presence of insecticides can reduce aphid phloem feeding relevant to persistent virus transmission in aphids and whiteflies. Active ingredients such have sulfoxaflor have been shown to reduce phloem feeding duration in the green peach aphid *Myzus persicae*, as well as reducing aphid ability to reach phloem. Additionally, seed treatments of thiamethoxam showed a reduction in probe duration and significantly reduced phloem feeding for the soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae). This reduction of phloem feeding has the potential to reduce persistent virus transmission and acquisition. Furthermore another commercial insecticide Sivanto 200SL® (flupyradifurone) reduced phloem ingestion in the whitefly.
*Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae), and was even able to reduce the transmission of the semipersistent tomato chlorosis virus (ToCV) \(^67\).

There have been previous studies assessing how current commercialized aphicides altered feeding behavior related to nonpersistent virus transmission in the green peach aphid, *M. persicae* \(^37\). These studies found no significant differences in the total number and duration of subphases II-1 and II-3 of potential drops within the first 5 minutes of aphids being placed onto the treated plants, which are related to nonpersistent virus transmission and acquisition respectively \(^37\). These initial EPG recordings were supported by the subsequent result demonstrating no significant changes in nonpersistent virus transmission after exposure to current commercialized aphicides \(^37\). However, there is no large-scale comparison of currently available commercial products on how they affect *A. gossypii* mortality and their propensity to alter feeding behavior related to persistent virus transmission. As a result of this, an understanding of how the commercial products affect *A. gossypii* feeding behavior relevant to virus transmission would be beneficial \(^37\).

**Chapter 1.5. Aphid Salivary Gland Physiology**

Aphid feeding on plant tissue relies on two major organs; the salivary glands and the pharyngeal-cibarial pump complex \(^46, 69\). The salivary gland is critical for feeding as it facilitates the secretion of saliva containing effectors that interact with plant defenses and prevent sieve tube plugging through the suppression of calcium defenses \(^69\). On the other hand, the pharyngeal-cibarial pump complex is essential in generating a sucking action during feeding \(^46, 70\). The essential nature of these organ systems permits the development of novel products that could disrupt these mechanisms to induce feeding cessation and mortality in aphids. An example of this is the commercialized insecticide pymetrozine, with studies providing evidence it prevents aphid fluid ingestion through the inhibition of the musculature
around the cibarial pump of sucking pests to prevent fluid ingestion \(^70\). Interestingly, there is currently a lack of commercial insecticides that directly target the aphid salivary gland as a control mechanism.

The comprehensive functions of saliva explain the importance of the aphid salivary gland in driving successful feeding events. The aphid salivary gland secretes two forms of saliva referred to as ‘watery’ and ‘gelling’ saliva, which facilitate enzymatic food digestion and the formation of the stylet sheath respectively \(^71\). The ‘watery’ salivary secretions of the aphids have been found to sabotage plant defenses and prevent sieve tube plugging by suppressing a rise in free calcium through molecular interactions of salivary proteins acting as a ‘calcium sink’ \(^69\). On the other hand, the ‘gelling saliva’ forms the salivary sheath, which is important in creating a seal around the mouthparts inserted into sieve elements to permit passive phloem ingestion \(^72\). Evidence for the importance of gel saliva in obtaining nutrients was demonstrated by reducing the amount of structural sheath proteins through RNA interference, which significantly reduced the efficacy of phloem feeding, thus affecting aphid survivorship \(^71\)-\(^73\). There is a possibility to develop products acting through novel mechanisms to reduce the secretory function of the salivary gland to both reduce aphid photosynthate removal and persistent virus transmission.

**1.6. Inward Rectifier Potassium (Kir) Channels Biology and Inhibitors**

One underexplored potential target for insecticides are the inward rectifier potassium (Kir) channels, which belong to a large ‘superfamily’ of K+ ion channels including calcium gated, voltage-gated, cyclic nucleotide-gated and two-pore channels \(^74\). On a molecular level, Kir channels consist of 4 subunits centered around a water-filled pore, where K+ ions cross the plasma membrane down their electrochemical gradient \(^75\). Each subunit consists of two transmembrane domains that contain intracellular NH2-terminal and COOH-terminal domains.
Each subunit has a H5 extracellular unit connecting the transmembrane segments, which form the K+ selectivity filter and contains the highly conserved GYG motif of K+ channels (Figure 1.2.). Kir channels alter the amplitude based on voltage by acting as a biological diode to transport K+ ions into cells at hyperpolarizing membrane potentials more than out of cells at depolarizing membrane potentials (Fig 1.2.). Importantly, a small amount of K+ travels out of the cell when the membrane potential (Em) is equal to the equilibrium of potassium (Ek), but Kir blockage occurs at depolarizing membranes when Em > Ek through magnesium (Mg2+) or polyamines (Fig. 1.2.). Multiple studies have found that inward flux of K+ ions at hyperpolarized membranes thorough Kir channels plays a critical role in mammalian and arthropod physiological systems, which include maintaining the resting membrane potential, cardiac and neuronal excitability, and epithelial transport.

Figure 1.2. Structure and function of inward rectifier Kir channels. (a) Behavior of nonrectifying K+ or Kir channels in plasma membranes. The nonrectifying channel mediates movement of K+ into or out of the cells depending on the direction and level of membrane polarization. The Kir channel mediates strong inward K+ movement at hyperpolarizing voltages but relatively weak or low outward movements of K+ at depolarizing voltages due to binding of intracellular Mg2+ to the channel pore, which blocks the outward movement of K+. Adapted from Piermarini et al. 75
It is important to understand the physiological mechanisms behind a potential organ system such as the salivary gland before creating antifeedant potential targets. For example, mammalian salivary glands support the resting secretion of salivary exocrine acinar cells through Kir channels driving anion efflux, whilst minimizing K⁺ ion loss. Furthermore, recent studies suggest Kir channels play important physiological roles in exocrine systems of dipteran insects. This evidence was supported by genetic deletion or pharmacological inhibition of Kir channels leading to a reduction in fluid and ion secretion of dipteran Malpighian tubules. Further, recent studies have demonstrated the critical role of Kir channels in arthropods arthropod salivary gland function for: the lone star, Drosophila, the Brown planthopper, horn flies and mosquitoes.

These studies above were permitted by a follow-up drug discovery campaign targeting the Anopheles gambiae Kir1 channel (AnKir1). A total of 17 sub-micromolar inhibitors of An. gambiae Kir1 were identified from the screening of 25,000 structurally diverse scaffolds. The most effective chemical discovered was VU041, which was shown to have an IC50 value of approximately 23.5 µM in voltage clamp electrophysiology studies on a cell line overexpressing the AnKir1. Importantly, VU041 was also determined to be one of the most selective to AnKir1, with no activity against other insect Kir channels. Additionally, phylogenetic studies for Kir cDNA within the soybean aphid Aphis glycines (Matsumara), discovered the presence of two distinct cDNA sequences in aphids. Phylogenetic comparisons found that Aphid Kir 2 (ApKir2) was evolutionary independent of other insect Kir2 channels, which indicates development of selective insecticides targeting ApKir2 is possible. This was supported by electrophysiological studies demonstrating that the Kir inhibitor VU041 inhibits A. glycines (soybean aphid) Kir1 and Kir2 receptors. Topical application of VU041 also resulted in acute mortality, which suggests Kir channels are
important for aphid survivorship. Further, a recent study by Li et al. found that foliar application of VU041 on cotton leaves prevented phloem feeding, and caused *A. gossypii* starvation in 48hr. The exact physiological mechanisms causing this change in feeding behavior are unknown, but it is suspected that VU041 is disrupting the secretory activity of gel saliva secretion that results in reduced salivary sheath length. These results taken in conjunction show the potential of VU041 as a potential novel mode of action antifeedant aphicide.

Unfortunately, the screening studies also found that VU041 demonstrated inhibitory activity against Kir2.1, which is expressed in the human heart and could result in adverse effects on heart function. This was remedied through the removal of the trifluoro group from the pyazole and replacement of the cyclohexane in VU041 with a tetrahydropyran group to generate the molecule termed VU730. VU730 lost activity toward Kir2.1, but is still highly potent toward AnKir1. VU730 has currently not been tested for its ability to effect salivary secretions in aphids or induce mortality by acting as an antifeedant insecticide.

### 1.7. Overarching Hypothesis to be Tested

Considering 1) cotton aphid ability to transmit over 50 viruses, 2) variations in commercial insecticides to control virus transmission 3) Kir modulator VU041 exposure preventing phloem feeding in *A. gossypii*, we hypothesized that biological and electrophysiological assays would provide a comprehensive summary of different commercialized aphicides ability to induce mortality and cause changes to aphid feeding behavior relevant to virus transmission. Secondly, we hypothesized Kir modulator VU730 will negatively impact aphid salivary secretions, ability to reach phloem and survivorship.
1.8. Objectives of the Study

The overarching goals of this study were to employ novel pharmacology, electrophysiology, and biological assays to create a baseline toxicity and feeding behavior measurement of commercialized aphids, which can be used to further validate Kir channels and Kir channel inhibitors as a novel aphid antifeedant target site and lead to chemical scaffolds amendable to development.

1. Test commercialized aphicides for toxicity and propensity to alter feeding behavior relevant to virus transmission

2. Determine the antifeedant capabilities of Kir modulators and our ability to translocate these lipophilic molecules within the plant

3. Develop and test if a novel solubilizer will increase systemic movement of Kir inhibitors that eliminate the need for foliar application
Chapter 2. Profile of Commercialized Aphicides to Survivorship and Feeding Behavior to the Cotton Aphid, *Aphis gossypii*

2.1. Introduction

Insects belonging to Order Hemiptera are one of the most numerous and damaging pests of agriculture due to their ability to remove photosynthate and horizontally transmit plant viruses \(^1\). Hemipterans transmit more than 70% of all known insect-borne plant viruses \(^{19, 86}\) and the cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), is one of the most destructive agricultural hemipteran pests that results in significant economic losses stemming from direct feeding damage of plant tissue or reduction of fruit development \(^{5-7}\).

Plant virus transmission occurs via distinct modes and correlated the virus location within the plant tissues \(^{19, 20}\). Persistent circulative or persistent propagative viruses are typically located within the plant vascular bundles (e.g. phloem sieve elements) and are acquired and transmitted over hours to days due to the latent period of the viruses \(^{19}\). In contrast, non-persistent viruses are located within the epidermal and mesophyll cells of the plant \(^{20, 22, 23}\) and are transmitted within seconds to minutes after intracellular punctures of these plant cells \(^{61, 87}\). These intracellular punctures occur due to aphids sampling sap during stylet movement within the plant and these punctures can be recorded and visualized as potential drops (pd) through Electrical Penetration Graph (EPG) electrophysiological recordings (Table 2.2.) \(^{50, 61}\). The salivation and subsequent ingestion of sap is related to non-persistent virus acquisition and transmission, respectively \(^{54, 62}\). The EPG technique also facilitates detection and monitoring of aphid sieve element salivation (E1) and phloem ingestion (E2) that are relevant to persistent virus transmission and acquisition, respectively \(^{19, 88}\) (Table 2.2.). Assessment of how commercial aphicidal products alter 1) the time between aphid landing and probing plant tissue, 2) the ability to reach xylem and phloem sieve elements, and 3) the duration of time spent within sieve elements are relevant to understanding how these products...
could affect aphid-mediated plant damage through photosynthate removal and horizontal transmission pathogen transmission.

Insecticides must kill the vector or alter aphid feeding behavior within 4-6 minutes to stop or limit acquisition and horizontal transmission of persistent or non-persistent plant viruses. However, some insecticides, such as pyrethroids, increase irritation prior to inducing lethality that leads to increased plant-to-plant movement and increased non-persistent plant virus transmission. Thus, understanding how insecticides alter aphid feeding behavior is important for the field of insecticide science and the development of pest management plans that aim to reduce aphid transmission of plant viruses.

*Acyrthosiphon gossypii*, as well as other aphid species, have evolved resistance to most commercialized aphicides and the global spread of aphicide resistance threatens the effectiveness of aphid control programs. This potential for reduced efficacy of commercialized aphicides has led to efforts to identify novel aphicides that alter feeding behavior and induce toxicity, yet there is a knowledge gap regarding how currently available commercial products differ in acute toxicity and changes to feeding to *A. gossypii*. Recent studies have demonstrated that some commercialized aphicides can reduce but not eliminate acquisition and horizontal transmission of persistent viruses, yet no commercial product has been shown to prevent the acquisition and transmission of non-persistent viruses. Quantification of alterations to aphid feeding behaviors that are relevant to virus transmission after exposure to commercialized aphicides will provide a benchmark for downstream development of novel aphicides. Thus, the goal of this work was to quantify the toxicity of commercially available aphicides and to observe their propensity to alter aphid feeding behaviors that are related to virus transmission.
2. MATERIALS AND METHODS:

2.2.1. Aphid colony maintenance

A laboratory colony of cotton aphid, *A. gossypii*, was developed from a single aptera collected from a cotton plant, *Gossypium hirsutum* L., at Louisiana State University Agricultural Center Macon Ridge Research Station, Winnsboro, LA, in 2006. The cotton aphid colony was reared on cotton variety DP1441RF (DeltaPine, Monsanto Company, St. Louis, MO, USA) and maintained in screened cages (BugDorm-1 Insect Rearing Cage, MegaView Science Co., Ltd., Taiwan) under laboratory conditions at 20 to 22°C and a photoperiod of 14:10 (L:D). All host plants were grown in an E-36L1 growth chamber (Percival Scientific Inc., Perry, Iowa) at 25°C and 50% RH, in 10-cm-diameter plastic pots filled with Miracle-Gro Potting Mix soil (Miracle-Gro Lawn Products Inc, Marysville, OH, USA) and Osmocote 14-14-14 (Scotts-Sierra Horticultural Products Company, Marysville, OH, USA) fertilizer. Fresh host plants were provided to the colonies every 2 to 3 weeks for colony maintenance.

2.2.2. Leaf dip toxicity assays

Eight different commercial insecticides with 5 different modes of action were used (Table 2.1.) and were tested in a leaf-disk dip-bioassay to observe relative toxicity at the highest recommended labeled rate for cotton. The concentration (PPM) of the active ingredient for each commercial insecticide used in this study is listed in Table 2.1 and was determined by calculating the percent active ingredient contained at the highest label rate with the minimum spray volume (15 gal/acre) for each product. Cotton plants were grown for 4-6 weeks prior to treatment at 26°C and 40% RH with a photoperiod of 14:10 (L:D). Cotton leaves were collected from the top of the plants where new growth was abundant. Leaves were cored with a no.149 arch punch (Osborne and Co., Harrison, NJ) to create cores of 11.34 cm². Leaf cores...
were dipped individually in a treatment or control solution of 100 ml for 10 sec. Cores were dried in a fume hood for 1 hour and placed into sterile Petri dishes (VWR polystyrene disposable, 200 X 15mm) on moistened Whatman 90 mm (#1) filter paper. One leaf disk was placed in a single Petri dish.

Each experiment consisted of two treatments, an insecticide treatment and a water treatment group (control). Ten cotton aphid adult apterae were placed on the leaf core that was treated with the aphicidal product or water (control). A total of three replicates per product was performed where one replicate consisted of an average toxicity across 10 leaf disks (n = 100 aphids per replicate). The Petri dishes were covered and placed in a growth chamber and mortality was assessed at 4-, 24-, and 72- hours after aphid infestation of the cores. Mortality was defined as a failure to elicit movement after manual prodding with a fine-tipped camel-hair brush. If products resulted in 100% mortality at 4 hours, a modified version of this method was utilized to assess mortality every 5 min over a 2 hour period at the same concentration of active ingredient (Table 2.1.).
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2.2.3. Probing behavior assays

Table 2.1. List of insecticides, the commercial product trade name, active ingredient, primary mode of action, and IRAC (Insecticide Resistance Action Committee) MoA number

<table>
<thead>
<tr>
<th>Trade Name</th>
<th>Active Ingredient</th>
<th>PPM of AI</th>
<th>Primary Mode of Action (MoA)</th>
<th>IRAC MoA Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grizzly Too®</td>
<td>Lambda cyhalothrin</td>
<td>305</td>
<td>Sodium channel modulators – Nerve action</td>
<td>3A - Pyrethroids</td>
</tr>
<tr>
<td>Brigade 2EC®</td>
<td>Bifenthrin</td>
<td>837</td>
<td>Sodium channel modulators – Nerve action</td>
<td>3A - Pyrethroids</td>
</tr>
<tr>
<td>Endigo ZC®</td>
<td>Thiamethoxam + Lambda cyhalothrin</td>
<td>296 + 394</td>
<td>Nicotinic acetylcholine receptor (nAChR) competitive modulators + Sodium channel modulators – Nerve action</td>
<td>3A - Pyrethroids + 4A - Neonicotinoids</td>
</tr>
<tr>
<td>Centric 40WG®</td>
<td>Thiamethoxam</td>
<td>499</td>
<td>Nicotinic acetylcholine receptor (nAChR) competitive modulators – Nerve action</td>
<td>4A - Neonicotinoids</td>
</tr>
<tr>
<td>Transform 50WG®</td>
<td>Sulfoxaflor</td>
<td>250</td>
<td>Nicotinic acetylcholine receptor (nAChR) competitive modulators – Nerve action</td>
<td>4C - Sulfoximines</td>
</tr>
<tr>
<td>Sivanto Prime®</td>
<td>Flupyradifurone</td>
<td>1246</td>
<td>Nicotinic acetylcholine receptor (nAChR) competitive modulators – Nerve action</td>
<td>4D - Butenolides</td>
</tr>
<tr>
<td>Fulfill®</td>
<td>Pymetrozine</td>
<td>687</td>
<td>Chordotonal organ TRPV channel modulators – Nerve action</td>
<td>9B - Pyridine azomethine derivatives</td>
</tr>
<tr>
<td>Movento 240 SC®</td>
<td>Spirotetramat</td>
<td>639</td>
<td>Inhibitors of acetyl CoA carboxylase – lipid synthesis, growth regulation</td>
<td>23 - Tetronic and Tetramic acid derivatives</td>
</tr>
<tr>
<td>Beleaf SG®</td>
<td>Flonicamid</td>
<td>699</td>
<td>Chordotonal organ modulators – undefined target site</td>
<td>29 - Flonicamid</td>
</tr>
</tbody>
</table>

The probing and feeding behavior of *A. gossypii* on untreated and insecticide treated plants were monitored using the EPG technique following our previously described methods. We aimed to test changes to aphid feeding behavior after exposure to commercial aphicides. The formulated products were mixed to the same concentrations used for toxicity assessments and treated plants were sprayed with a misting spray bottle (VWR, Avantor Sciences, Radnor, PA, USA) on the adaxial sides of the leaves. Plants were dried at room temperature for 1 hour prior to the EPG experiments. EPG experiments were performed using
a Giga8 DC amplifier (Wageningen Agricultural University, The Netherlands) with 1 gigaohm input resistance and an AD conversion rate of 100Hz running only the first four channels, which was performed in a Faraday cage. The analog signals were converted to digital signals using a DI-710 acquisition card (DATAQ Instruments, Inc., Akron, OH, USA). The signals were recorded and visualized using WinDaq Serial Acquisition software (DATAQ Instruments, Inc.). Apterous adult aphids were removed from cotton and immediately used in feeding behavior studies. A 2 cm length of 18-μm gold wire (Semiconductor Packaging Material, Armonk, NY, USA) was attached to the aphid dorsum with water-based silver glue. An individual aphid was infested on the adaxial side of a leaf of a single test plant (one aphid per plant) and a total of four plants treated with the same product were studied simultaneously. For assessments of percent feeding behaviors, a total of 10 replicates were performed where each replicate consisted of an average response across the four test plants. The final percent feeding behavior was determined by mean of the 10 replicates. Feeding behaviors were recorded for 7 hours.

Identification and classification of EPG signals followed the nomenclature of the list of EPG variables previously used by our group and others. Waveforms observed during EPG recordings and how they relate to aphid feeding behaviors within the plant are described in Table 2.2. The feeding behaviors we assessed were: 1) the proportion of aphids that successfully initiated a probe (with potential drop), 2) the time taken for that aphid to first initiate a probe (with potential drop), 3) the total time spent probing over a 7 h period for those aphids that probed on cotton, 4) the mean duration of each probe, 5) the proportion that continued to probe after 4 hr, 6) the proportion of aphids that successfully reached phloem, 7) the total time spent in phloem over a 7 hr for those aphids that reached sieve elements, 8) the mean duration spent in phloem for each probe, 9) the time taken from initial probe to first reaching phloem, 10) the total time spent salivating in sieve elements (E1 waveforms), 11) the
total time spent ingesting phloem (E2 waveforms), 12) and the proportion and duration of xylem feeding.

2.4. Data analyses

Aphid mortality was corrected using Abbott’s formula and the Arcsine square root transformation for all results displayed 92. Data was tested for normality using the Kolmogorov-Smirnov test in PROC CAPABILITY and tested for homogeneity using the Levene Test for Homogeneity of Variances in PROC GLM using SAS software version 9.3 (SAS Institute Inc. Cary, NC). Due to the assessment of aphid mortality at multiple time points, results were analyzed using a repeated measures ANOVA with Tukey’s HSD in PROC GLM SAS software version 9.3 (SAS Institute Inc. Cary, NC). The time to 50% lethality (LT50) values were generated using a nonlinear regression model (GraphPad Prism version 8.10 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com). Feeding behavior datasets and the percentage of aphids probing, salivating in sieve elements, or xylem (G waveforms) and phloem feeding (E2 waveforms) were tested for normality using the

<table>
<thead>
<tr>
<th>Waveform</th>
<th>Behavior description</th>
<th>Plant tissue location</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Stylet contact with leaf epidermis</td>
<td>Epidermis</td>
</tr>
<tr>
<td>B</td>
<td>Salivation on leaf epidermis and in mesophyll</td>
<td>Epidermis / mesophyll</td>
</tr>
<tr>
<td>C</td>
<td>Intercellular apoplastic stylet pathway</td>
<td>All tissues</td>
</tr>
<tr>
<td>PD</td>
<td>Intracellular punctures</td>
<td>Any living cell</td>
</tr>
<tr>
<td>E1</td>
<td>Salivation into phloem elements</td>
<td>Sieve elements</td>
</tr>
<tr>
<td>E2</td>
<td>Passive phloem sap uptake</td>
<td>Sieve elements</td>
</tr>
<tr>
<td>F</td>
<td>Derailed stylet mechanics</td>
<td>All tissues</td>
</tr>
<tr>
<td>G</td>
<td>Active intake of xylem sap</td>
<td>Xylem</td>
</tr>
</tbody>
</table>

values were generated using a nonlinear regression model (GraphPad Prism version 8.10 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com). Feeding behavior datasets and the percentage of aphids probing, salivating in sieve elements, or xylem (G waveforms) and phloem feeding (E2 waveforms) were tested for normality using the
Kolmogorov-Smirnov test (GraphPad Prism version 8.10, GraphPad Software, San Diego, California USA). Feeding behavior percentages that were not normally distributed were analyzed with the Kruskal-Wallis with Dunn’s multiple comparisons test to assess differences between the UTC and each of the commercial insecticides. Feeding behavior durations were first analyzed with the Brown-Forsythe test if Standard Deviations (SDs) were significantly different. Datasets with unequal SDs were analyzed with Welch’s ANOVA followed by Dunnett’s multiple comparisons test whereas datasets with equal SDs were analyzed with an ordinary one-way ANOVA followed by Dunnett’s T3 multiple comparisons test.

2.3. Results

2.3.1. Aphid leaf dip toxicity assay

Flupyradifurone, bifenthrin, and thiamethoxam + lambda cyhalothrin (labelled as tmx + lambda on the tables and figures) were the most toxic active ingredients tested with an average percent mortality of 99.7 ± 0.3%, 98.3 ± 0.8%, and 97.3 ± 0.8% at 4 hours post-exposure, respectively (Table 2.3.). Thiamethoxam and sulfoxaflor resulted in ≥ 90%, > 95% and 100% aphid mortality after 4-, 24-, and 72- hours post exposure, respectively (Table 2.3.). Lambda cyhalothrin, fonicamid, pymetrozine, and spirotetramat were less toxic with aphid mortality not reaching 50% at 4 hours post-exposure. At 72 hours post-exposure, fonicamid, spirotetramat, pymetrozine approached the threshold of 80% mortality but did not exceed it (Table 2.3.). Lambda-cyhalothrin was the least toxic at all time points with a maximum mortality of 41 ± 2.9% at 72 hours post-exposure, which was the longest time point studied.
Table 2.3. Percent mortality (± SE) of adult *A. gossypii* apterae in insecticide leaf dip assays over time

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>Active ingredient</th>
<th>4</th>
<th>24</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beleaf</td>
<td>Flonicamid</td>
<td>47.6 ± 1.8 c</td>
<td>47.9 ± 1.7 b</td>
<td>71.7 ± 1.5 b</td>
</tr>
<tr>
<td>Brigade</td>
<td>Bifenthrin</td>
<td>98.3 ± 0.8 ab</td>
<td>99.7 ± 0.3 a</td>
<td>100.0 ± 0.0 a</td>
</tr>
<tr>
<td>Centric</td>
<td>Thiamethoxam</td>
<td>93.7 ± 1.2 b</td>
<td>97.0 ± 1.3 a</td>
<td>99.7 ± 0.3 a</td>
</tr>
<tr>
<td>Endigo</td>
<td>Tmx + Lambda</td>
<td>97.3 ± 0.8 ab</td>
<td>98.0 ± 0.7 a</td>
<td>100.0 ± 0.0 a</td>
</tr>
<tr>
<td>Fulfill</td>
<td>Pymetrozine</td>
<td>13.7 ± 1.4 e</td>
<td>27.3 ± 2.3 c</td>
<td>64.5 ± 3.5 c</td>
</tr>
<tr>
<td>GrizzlyToo</td>
<td>Lambda-Cyhalothrin</td>
<td>5.4 ± 1.2 f</td>
<td>23.4 ± 2.5 c</td>
<td>41.0 ± 2.9 d</td>
</tr>
<tr>
<td>Movento</td>
<td>Spirotetramat</td>
<td>24.1 ± 2.6 d</td>
<td>46.2 ± 4.3 b</td>
<td>72.5 ± 3.5 b</td>
</tr>
<tr>
<td>Sivanto</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prime</td>
<td>Flupyradifurone</td>
<td>99.7 ± 0.3 a</td>
<td>100.0 ± 0.0 a</td>
<td>100.0 ± 0.0 a</td>
</tr>
<tr>
<td>Transform</td>
<td>Sulfoxaflor</td>
<td>93.7 ± 1.4 b</td>
<td>98.7 ± 0.6 a</td>
<td>100.0 ± 0.0 a</td>
</tr>
<tr>
<td>UTC</td>
<td>Untreated Control</td>
<td>0.0 ± 0.0 g</td>
<td>1.2 ± 0.3 d</td>
<td>5.5 ± 0.5 e</td>
</tr>
</tbody>
</table>

*Means followed by same letter within columns are not significantly different (P > 0.05; Tukey’s HSD).*

2.3.2. Assessment of Feeding Behaviors through EPG

2.3.2.1. Changes to probing behaviors

Aphid probing is defined as successful penetration of leaf epidermis that includes EPG waveforms A-C (pathway phase) and waveforms PD (potential drop). We aimed to assess the proportion of aphids that were able to probe on cotton after being placed on the leaf over the 7-hour EPG recording. Over the course of a 7-hour EPG recording period, flupyradifurone was the only active ingredient identified to alter aphid probing. Flupyradifurone reduced the
mean proportion of aphids that initiated a probe on cotton (with PD) to 37.5 ± 6.7% compared to 75.6 ± 7.9% of control aphids, which was a statistically significant ($P < 0.05$) reduction (Fig. 2.1.A.). Conversely, the presence of lambda cyhalothrin significantly increased the proportion of aphids that initiated a probe with potential drops from 75.6 ± 7.9% of control to 94.4 ± 3.7% (Fig. 2.1.A.), but not in a significant manner.

Of the aphids that probed, spirotetramat, flonicamid, flupyradifurone, bifenthrin, sulfoxaflor, and pymetrozine increased probe initiation rate by 2.7-, 10.2-, 2.8-, 5.8-, 6.8, and 2-fold, respectively (Fig. 2.1.B.). This data indicates that aphids infested on leaves exposed to the commercial aphicides listed above are initiating a first probe at a significantly ($P < 0.05$) faster significantly rate than the untreated control (Fig. 2.1.B.). Thiamethoxam increased the rate of probe initiation by 1.7-fold, which was not significantly different from control (Fig. 2.1.B.).

We assessed aphicide-induced changes to the total time spent in a probe, which includes waveforms pd, G, E1, and E2 throughout the 7-hour EPG recording period. Spirotetramat, pymetrozine, and lambda-cyhalothrin did not alter the time spent in probes when compared to control (Fig. 2.1.C.). However, leaf disks treated with flonicamid, thiamethoxam, flupyradifurone, bifenthrin, sulfoxaflor, or thiamethoxam + lambda cyhalothrin led to approximately 8-fold reduction of total probe duration compared to control treatments (Fig. 2.1.C.). The mean time spent in individual probes (Fig. 2.1.D.) mirrored the mean total probe duration with the exception of lambda-cyhalothrin that significantly reduced the mean time spent in individual probes. The lack of change for total probe duration with reduced individual probe times is likely due to the 2-fold increase in total number of probes after exposure to lambda-cyhalothrin (Fig. 2.1.E.).

The last assessment pertaining to probe behavior was to measure the percent aphids that probed after 4 hours of exposure. Flupyradifurone, bifenthrin, sulfoxaflor, thiamethoxam, and
thiamethoxam + lambda cyhalothrin yielded >90% mortality after 4 hours of exposure (Table 3) and a significant reduction of aphids that continued to probe after 4 hours was indeed observed (Fig. 2.1.F). However, specific focus was directed to aphicides that did not induce acute mortality to gain an understanding if feeding behavior was impacted, which could alter pathogen transmission dynamics. Pymetrozine, which was not acutely toxic, did not reduce the ability to probe after 4 hours (Fig. 2.1.F.). Importantly, flonicamid, which was not acutely toxic, significantly ($P < 0.001$) reduced the percentage that continued to probe after 4 hours to less than 10%, which is an 8-fold reduction when compared to control aphids (Fig. 2.1.F.).
Figure 2.1. Changes to aphid probing behavior as determined by EPG recordings over 7 hours for apterous cotton aphids fed on untreated or insecticide treated cotton plants. Bars represent mean duration or percent feeding behaviors are described on the Y-axis of graphs on each panel. Error bars represent SE. Asterisks represent statistically significant differences as determined by Kruskal-Wallace test followed by a Dunn’s multiple comparison test when compared to the control, with *, **, ***, and **** representing: $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively.
2.3.2.2. Proportion of aphids that reached phloem and feeding duration

Zero of 39 aphids were observed reaching phloem on Flupyradifurone-treated leaves and only 1 of 39 aphids reached phloem for a duration of 160 seconds within a 7-hour recording period when feeding on flonicamid-treated plants, which were significantly \((P < 0.0001)\) reduced to control treatments (Fig. 2.2.A.). The proportion of aphids that reached phloem was significantly reduced for thiamethoxam (4.5-fold), bifenthrin (5.9-fold), sulfoxaflor (6.5-fold), and thiamethoxam + lambda cyhalothrin (4-fold) when compared to control treatments (Fig. 2.2.A.). Of the aphids that reached phloem, the total time spent in phloem and mean phloem feeding per probe were significantly \((P < 0.05)\) reduced for thiamethoxam, sulfoxaflor, and thiamethoxam + lambda cyhalothrin (Figs. 2.2.B. and 2.2.C.). Yet, the mean duration aphids spent in phloem was increased by 2.3-fold for leaves treated with pymetrozine, which is a significant \((P < 0.05)\) increase when compared to control (Fig. 2.2.C.). Of those aphids that reached phloem, thiamethoxam, bifenthrin, and thiamethoxam + lambda cyhalothrin significantly reduced the time required to move from initial probe to reaching phloem elements by 2.7-, 5.2-, and 3.2-fold, respectively (Fig. 2.2.D.). It was not possible to quantify and compare phloem feeding duration, E1 (sieve element salivation), or E2 (phloem sap ingestion) for Flupyradifurone- or flonicamid- treated leaves due to the lack of aphids that reached phloem.

2.3.2.3. Time spent in sieve element salivation, phloem ingestion, and xylem ingestion

Next, we aimed to test how commercialized aphicides impacted duration of sieve element salivation (E1) and passive phloem ingestion (E2). Time spent in E1 and E2 was significantly \((P < 0.05)\) reduced when aphids were infested on thiamethoxam, sulfoxaflor, and thiamethoxam + lambda cyhalothrin treated leaves (Figs. 2.2.E. and 2.2.F.). Yet, bifenthrin and pymetrozine increased time spent in E1 and E2 that indicates these two products increased
sieve-elements salivation (E1) and phloem ingestion (Figs. 2.2.E and 2.2.F), although this difference was not significant.

In addition to phloem sieve elements, we tested if these products alter the ability of aphids to reach xylem sieve elements (waveform G). Flupyradifurone and flonicamid significantly ($P < 0.01$) reduced the proportion of aphids that reached xylem by 3.3- and 1.9-fold, respectively, when compared to control (Fig. 2.2.G). The total time aphids spent in xylem was significantly reduced for all commercial products except for spirotetramat, lambda cyhalothrin, and pymetrozine (Fig. 2.2.H).

Figure 2.2. Vascular bundle feeding and probing parameters as determined through EPG recordings across 7 hours for apterous cotton aphids fed on untreated or insecticide treated cotton plants. Bars represent mean total duration or the percent feeding behaviors described on the Y-axis of graphs on each panel. Error bars represent SE. Asterisks represent statistical significance where *, **, ***, and **** represent $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively.
2.3.3. Time dependent toxicity of select aphicides

The toxicity of flupyradifurone, bifenthrin, and thiamethoxam + lambda led us to compare time dependent toxicity of these three molecules through leaf-dip toxicity bioassays. Flupyradifurone induced 90% mortality within 30 min and 100% mortality within 45 min that resulted in an LT$_{50}$ of 8.9 min (95% CI = 4-8, r$^2$: 0.98; Fig. 2.3.A.). Bifenthrin induced 90% mortality within 60 minutes and an LT$_{50}$ of 32.1 min, (95% CI: 30-34, r$^2$: 0.96; Fig. 2.3.A.). Lastly, thiamethoxam + lambda was the least toxic of the three and induced 90% mortality within 55 min and resulted in an LT$_{50}$ of 30.3 min (95% CI: 28-32, r$^2$: 0.97; Fig. 2.3.A.). The onset of 90% mortality after 30 minutes of exposure to flupyradifurone was supported by the EPG data that suggested only 10.0 ± 4.1% of the aphids placed onto cotton treated with flupyradifurone initiated probes after 30 min of infestation on the treated leaf (Fig. 2.3.B.).

![Figure 2.3](image)

**Figure 2.3.** Effects of select aphicides to *A. gossypii* survivorship and probing behavior. A) Toxicity of aphids after contact exposure to flupyradifurone-, bifenthrin-, or thiamethoxam + lambda cyhalothrin-containing products on leaf tissue at 5 min intervals across 60 minutes. Data points represent mean (n = 75) percent toxicity at each time point with error bars representing standard error (SE). B) Changes to probing behavior after contact exposure to flupyradifurone (Sivanto Prime) compared to control.

2.4. Discussion

This study aimed to compare toxicity and changes to aphid feeding behavior after exposure to commercialized aphicides to establish a baseline dataset of comparative toxicity and the potential of these products to alter aphid-vectored virus transmission through behavior.
changes. It is accepted that toxicity of commercialized aphicides to should be greater than 80% at 4 hours post-exposure to be considered relevant for field reduction of aphids. Based on this threshold, only the bifenthrin-, thiamethoxam-, thiamethoxam + lambda cyhalothrin-, flupyradifurone-, and sulfoxaflor-containing aphicides would be considered effective (Table 2.3.). Yet, flonicamid, pymetrozine, and spirotetramat are considered to be effective aphicides within the agricultural community despite the inability to reach 80% mortality within 4 hours of exposure (Table 2.2.-2.3.). Thus, while acute toxicity is an obvious and desirable endpoint to reduce infestations and aphid-vectored plant pathogens, we contend that mitigation of aphid-mediated damages to plant health control can be achieved through other biological endpoints, such as reduced plant feeding and/or inhibition of probing behavior. To this point, flonicamid has been described to have antifeedant activity that likely led to the near elimination of probing behavior after 4 hours of exposure despite low acute toxicity (Fig. 2.1.D., Table 2.3.).

The impact of aphicides to alter the ability of individual aphids to phloem feed is a relevant factor that should be considered for insect survival through their ability to acquire nutrients and for transmission of persistent viruses. Previous reports highlight the ability of select aphicides to reduce aphid phloem feeding, which will presumably correlate to a reduction of horizontal transmission and acquisition of persistent viruses. This study expands on previous work by providing evidence that commercialized aphicides containing flonicamid, nAChR modulators (sulfoxaflor and flupyradifurone, and thiamethoxam), pyrethroids (bifenthrin), and a combination of the two (thiamethoxam + lambda-cyhalothrin) disrupt aphid ability to reach phloem and decrease phloem feeding duration (Figs. 2.2.A. and 2.2.B.), which could potentially reduce persistent virus acquisition and transmission. This was supported in a recent paper demonstrating that flonicamid treated plants were able to significantly reduce the acquisition rate and subsequent inoculation of the phloem-restricted luteovirus turnip yellows virus for *Myzus persicae* Sulzer (Homoptera: Aphididae). Further,
sulfoxaflor has been shown to invoke feeding cessation, reduced ability to find phloem, and duration of phloem feeding in the green peach aphid, *M. persicae* 44, 65. Lastly seeds treated with thiamethoxam showed reduced total probe duration with potential drop and significantly reduced phloem feeding on the soybean aphid, *Aphis glycines* (Hemiptera: Aphididae), which could also reduce plant nutrient loss and persistent virus transmission 66. Additional downstream studies are required to test this speculation of whether these aphicides will reduce *A. gossypii* persistent virus transmission.

The combination of leaf dip toxicity assay results and the analysis of EPG data suggests that Sivanto Prime® (flupyradifurone), Transform 50WG® (sulfoxaflor), Centric 40WG® (thiamethoxam), Endigo ZC® (thiamethoxam + lambda cyhalothrin), Brigade 2EC® (bifenthrin), and Beleaf SG® (flonicamid) are likely to provide effective control of susceptible cotton aphids and contribute to the reduction of extended aphid phloem feeding 19 (Table 2.3., Fig. 2.2.A.-C.). This is supported by a 2019 field study that also found significant reductions in aphid populations compared to an untreated control for products containing flupyradifurone, sulfoxaflor, and thiamethoxam 95. On the other hand, we have shown that spirotetramat-, lambda cyhalothrin-, and pymetrozine-containing products resulted in low mortality and did not significantly alter feeding behavior, suggesting these products may not provide effective control of field populations of cotton aphids based on current benchmarks 93. In addition, our data indicate *A. gossypii* were highly susceptible to bifenthrin, yet previous reports have reported low toxicity to *A. gossypii* after treatment of field plots with the bifenthrin-containing product, Brigade 2EC® 95. The differences in toxicity likely result from our study using a lab colony that has not been selected with insecticidal pressures in 16 years versus a field population that are likely to possess resistance mechanisms to pyrethroid insecticides.

The flupyradifurone-containing aphicide (Sivanto Prime) was the most toxic product studied (Fig. 2.3.A.), the most effective product at reducing the proportion of aphids that
initiated a probe (Fig. 2.1.), and most effective inhibitor of phloem feeding at the tested concentrations (Fig. 2.2.). These data indicate flupyradifurone combines desirable endpoints of antifeedant activity with rapid toxicity. These data support previous studies that have shown that another commercial insecticide Sivanto 200SL® (flupyradifurone) reduced phloem ingestion 1 in the whitefly *Bemisia tabaci* and reduced transmission of the semipersistent tomato chlorosis virus 67. Preventing transmission of non-persistent viruses is challenging due to horizontal transmission occurring between 4-6 minutes of probing 36 and unfortunately, the time to >90% mortality after contact exposure to flupyradifurone through leaf dip bioassay was >30 minutes (Fig. 2.3.A.), which is 6-8-fold longer than the time for inhibition of non-persistent pathogen transmission. However, studies directed to quantifying changes to virus transmission in plants treated with product in a field relevant manner are warranted to substantiate these data. Regardless, flupyradifurone was the most effective active ingredient studied this this work and represents a benchmark for newly developed aphicides. Opposite of flupyradifurone, lambda cyhalothrin increased probing behavior (Fig. 2.1.A.) and, when combined with increased plant-to-plant movement after probing, likely explains the observed increase in the spread of non-persistent, stylet-borne viruses within and across agricultural fields 28, 29, 31.

This study provides the first evidence that commercial products containing spirotetramat, flonicamid, flupyradifurone, bifenthrin, sulfoxaflor, and pymetrozine cause *A. gossypii* to initiate the first probe with potential drops on cotton faster than an untreated control plant (Fig. 2.1.B.). The increased rate of potential drops after landing on plant tissue could be due to gustatory signaling pathways that driving feeding. Host plant selection by aphids involve a variety of sensory and behavioral mechanisms for both host plant location and recognition 96. Once the aphid has landed on the plant surface prior to probing on the epidermis, a variety of surface cues, such as plant volatile and gustatory cues, can be assessed prior to
stylet insertion as the aphid walks on the plant surface \(^{96-99}\). Considering we analyzed formulated products, we speculate that formulation of these products stimulates feeding cues to increase probe rate that would lead to increased rate of toxicity. While this approach is ideal for acutely toxic molecules, stimulation of probing or other feeding behaviors for molecules that induce mortality through antifeedant mechanisms and not acute toxicity could reduce product efficacy.

In conclusion, this research highlights considerable differences in relative toxicities of commercialized aphicides in lab-based experiments and select aphicides significantly alter multiple aspects of aphid feeding biology. Of significant interest is the notion that altering feeding behavior in the absence of toxicity can result in effective aphicides, which is evidenced by flonicamid. This study defines endpoints related to *A. gossypii* survivorship and feeding behavior after exposure to commercialized aphicides that can be used to compare newly developed aphicide chemical scaffolds and modes of action that aim to prevent plant infestation and transmission of non-persistent or persistent viruses.

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2.6. Notes

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Chapter 3. Catalyzing systemic movement of inward rectifier potassium channel inhibitors for antifeedant activity against the cotton aphid, *Aphis gossypii* (Glover)

3.1. Introduction

Aphids are polyphagous hemipteran pests that contribute to economic losses exceeding $1 billion annually \(^2\)\(^-\)\(^4\). These losses result from feeding events that reduce yield through photosynthetic removal and/or plant virus transmission \(^9\)\(^-\)\(^12\). Effective control of aphid populations has relied heavily on the use of synthetic insecticides, yet development of insecticide resistance through target-site mutations \(^100\), \(^101\) and enhanced metabolic detoxification \(^100\), \(^102\), \(^103\) has reduced the efficacy of most commercialized insecticide classes to control aphids \(^104\), \(^105\). The established resistance to commercialized aphicides \(^105\)\(^-\)\(^107\) has provided the impetus to identify novel chemical scaffolds and novel target sites that can be developed to complement current aphid control strategies.

Aphid probing and feeding on plant tissue is the biological event responsible for aphid-induced plant damages within agricultural systems and is dependent on proper function of the salivary glands. The salivary gland is the tissue driving pathogen acquisition and transmission and the primary salivary gland is responsible for secretion of gelling and the accessory salivary gland secretes watery saliva that form the stylet sheath and facilitate food ingestion, respectively \(^108\). Thus, our previous work \(^109\) aimed to identify physiological pathways critical to the functionality of the aphid salivary gland and test the toxicological relevance by developing chemical inhibitors to prevent salivary gland function and subsequent feeding events. Data indicate inward rectifier potassium (Kir) channels regulate the secretory activity of the salivary gland and subsequent feeding of sap-feeding and hematophagous arthropods \(^109\)\(^-\)\(^115\). More specifically, the insect-specific Kir channel inhibitor, VU041, reduced salivary secretions and prevented sieve element salivation and phloem ingestion in the cotton aphid, *A. gossypii* \(^109\). These data provided the first insight into the functional role of Kir channels in
the aphid salivary gland and suggested aphid Kir channels are promising targets for development of novel antifeedants and toxicants.

The increased concern for chemical exposure to non-target organisms (e.g., pollinators) and environmental contamination has led to prioritization of plant systemic insecticides that are toxic to herbivorous insects after chemical uptake by the roots or leaves and translocated to all parts of the plant through sieve elements. Lipophilic insecticides, such as VU041 and other Kir inhibitors, are poorly water-soluble and precipitation in water severely limits incorporation into various platforms commonly employed for increasing plant systemic movement. Thus, lipophilic insecticides are usually restricted to non-systemic delivery mechanisms, such as foliar treatment of plant tissue or contact exposure of aphids. Although commercial solubilizers exist to increase plant systemicity, currently available solubilizer products only create partially soluble suspension concentrates (SC) or emulsifiable concentrates (EC) that restrict methods for chemical delivery to foliar applications through sprays. Although clear and transparent, SC or EC becomes translucent or opaque upon dilution with water, which reduces the treated surface area by up to 2,500 times when compared to transparent solutions. To circumvent this, we have invented a water-soluble concentrate (WSC) that enables water solubility of lipophilic molecules to promote plant systemicity.

Considering Kir channel inhibitors reduced salivary gland function, aphid feeding, and survivorship as well as the need for development of a novel delivery system to enable systemic movement of Kir modulators, we aimed to 1) employ a multidisciplinary approach to further validate Kir channels as a novel aphid antifeedant target site and 2) develop and test if Kir Inhibitor WSC (KI-WSC) will increase plant systemicity to enable translocation and translaminar movement of Kir inhibitors. Knowledge gained from this study may be used to broadly guide future development of Kir directed antifeedants and provide proof-of-concept.
for enabling water solubility and plant systemicity of lipophilic insecticides that can be used to mitigate economic losses resulting from feeding of sap sucking agricultural pests.

3.2. Materials and Methods

3.2.1. Compounds, reagents, and insects

The Kir channel inhibitors VU041 \((1-(3,4\text{-dihydroquinolin-1(2H)-yl})-2-(3\text{-}(\text{trifluoromethyl}-4,5,6,7\text{-tetrahydro-1H-indazol-1-yl})\text{ethan-1-one})\) and VU730 were originally identified in high-throughput screens to identify inhibitors of \(Anopheles gambiae\) Kir1 \(^{117}\). Both compounds were custom synthesized and purchased from Molport Inc. (Riga, Latvia) and were purified by column chromatography (>95% pure by \(^1H\) NMR analysis). The molecular structures of VU041 and VU730 are shown in Figure 2.1. Rhodamine B was purchased from Sigma-Aldrich (St. Louis, MO, USA). The laboratory colony of \(A. gossypii\) was derived from a single aptera aphid found at Louisiana State University Agricultural Center Macon Ridge Research Station, Winnsboro, LA, in 2006 and was collected from a cotton plant, \(Gossypium hirsutum\) \(^{109}\). The cotton aphid colony was reared as we previously described \(^{109}\) on cotton, \(Gossypium hirsutum\) \(^L\). variety DP1441RF (DeltaPine, Monsanto Company, St. Louis, MO, USA). All host plants were grown in 10-cm-diameter plastic pots filled with Miracle-Gro Potting Mix soil (Miracle-Gro Lawn Products Inc, Marysville, OH, USA) and Osmocote 14-14-14 (Scotts-Sierra Horticultural Products Company, Marysville, OH, USA) fertilizer at 25°C and 50% RH in an E-36L1 growth chamber (Percival Scientific Inc., Perry, Iowa) and a photoperiod of 14:10 (L: D). All colonies were reared in isolation from any known pathogens.
3.2.2. Generation of water-soluble concentrate for VU041 and VU730

The solubilizer used for this study was rubusoside. Rubusoside was isolated from Rubus suavissimus S. Lee (Rosaceae)\textsuperscript{118, 119} and structurally elucidated by NMR and MS analyses. The purity of rubusoside was determined to be above 98% by HPLC–UV. Appropriate amounts of the solubilizer (rubusoside) and a Kir modulator (VU041 or VU730) were weighed and mixed at the ratio of 100:1 weight/weight (VU041) or 100:2 weight/weight (VU730). Ethanol was added to the mixture at volume by weight ratio of 100:5 and the continuum was vortexed vigorously to dissolve and create a clear ethanol solution. This ethanol solution was passed through 0.45 µM nylon filters (Whatman, Maidstone, UK) to eliminate large particles present in the solution. The ethanol solution was allowed to stand in the room temperature for 60 min for stability observation. The ethanol in the solution was then evaporated under reduced pressure, heat at 50°C, and agitation in a RAPIDVAP system (Labconco, Kansas City, MO). Once the solvent was evaporated, deionized water was added to reconstitute the solid residue to a water solution of VU041 or VU730. This became the WSC of VU041 (VU041-WSC) or VU730 (VU730-WSC). The particle size of VU041-WSC and VU730-WSC in water was measured by a dynamic light-scattering (DLS) apparatus (Microtrac Nanotrac 251 ULTRA, Montgomeryville, PA, USA) at 25°C and an angle of 90°.

3.2.3. Salivary sheath measurements using artificial host assay

An artificial host feeding assay was used to measure the effects of Kir channel inhibitors on saliva secretions through changes of salivary sheath lengths, which we previously described\textsuperscript{109}. Kir modulators were solubilized in DMSO and diluted into the 15% sucrose solution with final DMSO concentrations not exceeding 0.1%. VU730 was studied at concentrations of 1mM, 700µM, 500µM, and 300µM when compared to control of 15% and 0.1% DMSO.
Individual, synchronized apterous adult aphids were all starved for 2 hours placed into the feeding chamber and provided access to the sucrose solution for 24 hours in an incubator at 22°C and a photoperiod of 14:10 (L: D). Analysis of sheath length measurements were performed immediately upon aphid removal. To enable identification and accuracy of salivary sheath measurements, we included the fluorophore, Rhodamine B, in the sucrose solution at a concentration of 200 ppm. The presence of fluorescent mouthparts or bodies was verified to ensure aphids included in the data analysis attempted to feed on the solution and non-fluorescent aphids were not included in data analyses. The Parafilm membrane was removed after 24 hours of feeding and the membrane was mounted on a microscope slide and measured under a Leica DM6B microscope. Sheath lengths from each concentration were averaged between 10-15 individuals and a concentration-response curve was constructed to determine the effective concentration to reduce sheath length by 50% (EC\textsubscript{50}).

3.2.4. Toxicity bioassays

Cotton plants were grown for 4-6 weeks prior to treatment at 26°C and 40% RH with a photoperiod of 14:10 (L: D). Toxicity bioassays were performed through foliar treatment of cotton leaves with Kir inhibitors solubilized in DMSO and diluted into PBS. Kir inhibitors were solubilized in DMSO, diluted into water at 1mM (0.1% DMSO) and 500 µL of solution was pipetted onto the leaf at a final concentration of 2 µg/cm\textsuperscript{2}. Treated leaves were dried for 24 hours at room temperature. To prevent cytotoxicity, final DMSO concentrations did not exceed 0.1% and control treatments included 0.1% DMSO. Five apterous adult aphids were placed into a single clip cage (1.5 cm x 1.5 cm x 0.25 cm) that contained a fine mesh to prevent aphid escape and was lined with cotton on the underside of the leaf. A total of 8 replicates were performed with 5 aphids per replicate that totaled 40 adult aphids per treatment group. Percent mortality for each clip cage was assessed at 4-, 24-, 48-, 72-, and 96- hours and dead
aphids were defined by lack of movement within 10 seconds after prodding with a fine camel-hair brush.

Aphid mortality after exposure to plants treated with VU041-WSC and VU730-WSC was measured through a leaf-disk bioassay. The KI-WSC (5 mg) was pipetted on the adaxial side of a single leaf at the bottom of the plant and manually spread across the leaf surface with a paint brush. The paint brush was cleaned with 70% ethanol between each plant to ensure concentrations painted onto the leaf remained consistent. Plants were left at room temperature to dry for 2 hours and returned to the E-36L1 environmental chamber (Percival Scientific Inc., Perry, Iowa) to incubate for 72 hours. Cotton leaves were collected from the top of the plants where new growth was abundant and did not include the treated leaf. These cotton leaves were an average distance of 10 – 12 cm from the treated leaf along the stem to the upper leaf. Leaf cores were taken with a no.149 arch punch (Osborne and Co., Harrison, NJ) to create cores of 11.34 cm². Each experiment consisted of two treatments: a leaf from a KI-WSC treated plant and an WSC-only control. Individual leaf cores were placed on moistened Whatman 90 mm (#1) filter paper in sterile Petri dishes (VWR polystyrene disposable, 200 X 15 mm). Ten adult apterae aphids were placed on a single leaf core, covered to prevent aphid escape, and placed in the environmental growth chamber. Aphid mortality was averaged across a total of 10 leaf disks (10 aphids per disk) at 4-, 24-, 72-, and 96- hours after aphid infestation of leaf cores. Aphid mortality was assessed through prodding individuals with a fine-tipped camel-hair brush and “dead” was defined as those that failed to show movement within 10 seconds of prodding.
3.2.5. Electrical Penetration Graph Recordings

3.2.5.1. General EPG Methods

Apterous adult aphids were removed from colony maintenance cotton plants and immediately used in EPG studies. A 2 cm length of 18-μm gold wire (Semiconductor Packaging Material, Armonk, NY, USA) was attached to the aphid dorsum with water-based silver glue. Four aphids were tested simultaneously, one aphid per plant. EPG experiments were performed with a Giga8 DC amplifier (Wageningen Agricultural University, The Netherlands) with 1 gigaohm input resistance and an AD conversion rate of 100 Hz running only the first four channels, which was performed in a Faraday cage. The analog signals were converted to digital signals using a DI-710 acquisition card (DATAQ Instruments, Inc., Akron, OH, USA). Signals were recorded and visualized using WinDaq Serial Acquisition software (DATAQ Instruments, Inc.). Identification and classification of EPG signals followed the nomenclature of the list of EPG variables previously used by our group and others. Signals observed during EPG recordings and how they relate to aphid feeding behaviors within the plant have been categorized as waveforms A-G and PD in previous studies. Waveforms A and B refer to stylet contact with the leaf epidermis and salivation with the epidermis and mesophyll, respectively. Waveform C is related to intercellular apoplastic stylet pathway in all plant tissues, whilst potential drops (PD) are intracellular punctures that occur within any living cell when aphids take sap samples. Aphid mouthparts reaching sieve elements (waveform E) can be broken down into two distinct subphases, which are sieve-element secretion of watery saliva (E1) and passive phloem ingestion (E2). Pattern E2 is always preceded by E1, which would indicate that the secretion of saliva in sieve elements (E1) is required for passive phloem ingestion (E2). Lastly, Waveform F indicated derailed stylet mechanics in all tissues and waveform G indicates the active intake of xylem sap. In total, we scored the following feeding behaviors: the proportion of aphids that successfully probed...
(with potential drop), the time taken for that aphid to first initiate a probe (with potential drop),
the total time spent probing over a 7 hour period, the total number of probes with potential
drops that occurred for each aphid, the mean duration of each probe, the proportion of aphids
that successfully reached phloem, time required for an aphid to successfully reach phloem, the
total time spent in phloem over 7 hours for those aphids that reached sieve elements, the mean
duration spent in phloem for each probe, and the proportion and duration of xylem feeding of
those that reached the xylem.

3.2.5.2. EPG analysis after Foliar Treatment

Foliar application of Kir inhibitors followed our previously described procedures and
recordings were performed across a 4 hour continuous window with a total of six replicates
consisting of 24 aphids per replicate. To test the antifeedant activity of VU730 after foliar
treatment, 500 µL of 1 mM VU730 was applied to the adaxial side of the leaf following our
previously described methods and left to dry for 24 hours prior to EPG analyses.

3.2.5.3. EPG analysis with systemic KM-WSC

A schematic diagram illustrating methodology for treatment of leaves with VU041-
WSC and VU730-WSC and the proposed translaminar and translocalized movement is shown
in Figure 3.1.B.-C. To establish whether incorporation of VU041 and VU730 with WSC
permitted plant systemic movement, we tested if antifeedant capabilities of KI-WSC treated
plants was maintained for aphids probing on the underside of treated leaves (trans-laminar) and
on the untreated upper leaves (translocation). To test for translocation of VU041-WSC or
VU730-WSC, one lower leaf per plant (Fig. 3.1.C., arrow) was immobilized and KI-WSC (5
mg) was pipetted onto the adaxial side of the leaf and distributed across the leaf surface with a
paint brush and left to dry for 2 hours. The treated cotton plants were incubated for 72 hours

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in screened cages at 22°C and a photoperiod of 14:10 (L: D). After incubation, individual apterous aphids were placed onto the adaxial side of a leaf at the top of the plant (Fig. 3.1.C.) and EPG recordings were performed across seven hours. A total of eight replicates were performed with a total of 32 aphids per replicate.

For trans-laminar experiments, one lower leaf per plant was immobilized and 2.5 mg of KI-WSC was pipetted onto the adaxial side of the leaf, which was then distributed across the leaf surface with a paint brush and left to dry for 2 hours. Any plants where KI-WSC contacted the abaxial side of the leaf was discarded. The treated cotton plants were incubated for 24 hours in screened cages in laboratory conditions at 22°C and a photoperiod of 14:10 (L:D) before the plants were utilized in EPG recordings. After incubation, the abaxial side of a leaf at the top of the plant was infested with individual aphids and EPG recordings were performed across seven hours. A total of eight replicates were performed with a total of 32 aphids per replicate.

Fig. 3.1. Diagrammatic representation of WSC-permitted translaminar and translocalized movement of VU041 and VU730 within the plant. A) Chemical structures of Kir inhibitors used in this study dissolved in WSC (KI-WSC) and applied to adaxial surface of the leaf. B) Cross section shows KI-WSC translaminar movement (blue arrows) through upper leaf epidermis to lower leaf epidermis. C) Translocalized movement (red arrows) of KI-WSC from adaxial leaf surface to leaf vascular bundle (xylem and phloem), which transports KI-WSC to upper leaves of the plant.
3.2.6. Liquid Chromatography Mass Spectrometry (LC-MS) for Quantification of Kir modulators in the upper leaf after Kir translocation

Leaves that were used in the EPG translocation trials were placed in labeled plastic bags and stored at -20°C immediately after the EPG experiments. Five different leaves from five different plants were used for LCMS analysis of VU730 and VU041, which were compared to their respective WSC-treated plants as a negative control. Leaf tissue was prepared using adjusted methods from a previous study \(^{120}\) prior to analysis by mass spectrometry. Strips of leaf tissue 2 mm in length were cut perpendicular to the leaf stem and 5ml of methanol (MeOH) was added to 0.5 g of leaf tissue from a single leaf. Falcon tubes containing MeOH soaked leaf tissue were vortexed for 30 seconds before leaf tissue was homogenized using a Sonic Dismembranator Model 500 probe (Fisher Scientific, Pittsburgh, PA, USA) for 30 seconds. Falcon tubes were filled to 10ml with MeOH before being vortexed for 30 seconds and centrifuged for 30 minutes at 4000 RPM. Following this centrifugation, 1ml of supernatant was removed was diluted (50:50) with 0.1% Formic Acid. Each individual sample was dried overnight in a Speed Vac prior to being re-solubilized in 100µl of MeOH and centrifuged for an additional 10 min at 10,000 RPM. Supernatant was placed into the autosampler vials and loaded into an HPLC 1260 Infinity II High Performance Liquid Chromatography (Agilent, Santa Clara, CA, USA). The HPLC was used to introduce 20 µl of sample to the source of Electrospray Ionization (ESI) TOF (Time-of-flight) 6230 (Agilent, Santa Clara, CA, USA). Thus, identification of the [M+H] of each compound was made using retention time and exact mass. Ion intensity vs. mass-to-charge ratio (m/z) was obtained for each compound. The exact mass for the M+H of VU730 and VU041 were calculated to 3 d.p. using the Mass calculator function in MassHunter software (Agilent, Santa Clara, CA, USA), which were 298.156 m/z and 364.163 m/z, respectively.
2.8 Data analyses

Sheath lengths from each treatment group were averaged between 10 and 15 individuals for 3 replications and were statistically analyzed by a Welch’s ANOVA with Dunnett’s T3 multiple comparisons test (GraphPad Prism version 9.3.1 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com). Concentration-response curves were generated for VU730 through testing of 5-7 concentrations and the concentration to reduce sheath length by 50% (IC$_{50}$) for VU730 was determined through a nonlinear regression model using GraphPad Prism (GraphPad Software, San Diego, California USA). Feeding behavior datasets and the percentage of aphids probing, xylem and phloem feeding were tested for normality using the Kolmogorov-Smirnov test using GraphPad Prism (GraphPad Software, San Diego, California USA). Feeding behavior and percentages were not normally distributed, therefore a Mann-Whitney U test was used to compare feeding behaviors between the UTC and Foliar application of VU730. For KI-WSC data, Arcsine square root transformation was performed for all the percentages of select feeding behaviors followed by an Ordinary one-way ANOVA with Tukey’s multiple comparisons test. The durations of different feeding behavior were initially analyzed with the Brown-Forsythe test assess whether Standard Deviations (SDs) were significantly different. Datasets with unequal SDs were analyzed with Welch’s ANOVA followed by Dunnett’s T3 multiple comparisons and data sets with equal SDs were analyzed with an ordinary one-way ANOVA followed by Tukey’s multiple comparisons test.

3.3. Results

3.3.1. VU730 reduces salivary secretions of *A. gossypii*

Aphids secrete gelatinous saliva from their salivary glands that forms salivary sheaths required for phloem feeding and thus, the length of the salivary sheath is a direct measurement of the secretory activity of the aphid salivary gland. Control aphids secreted salivary sheaths
with a mean length of $50.1 \pm 4.2 \, \mu m$ and exposure to VU730 led to a concentration-dependent reduction of salivary sheath length. Inclusion of 1 mM, 700 μM, or 500 μM VU730 into 15% sucrose solution significantly reduced the secretory activity by 3.3-, 1.9- and 1.6-fold respectively (Fig. 3.2.A.) when compared to control. The concentration to reduce 50% of the secretory activity (EC$_{50}$) was 691.2 μM, (95% CI: 569.1-881.5; Hillslope: -2.0; r$^2$:0.98 (Fig. 3.2.B.) and representative images of salivary sheaths embedded into the Parafilm membrane for control and VU730 treated aphids are shown in Figs 3.2.B.-3.2.C., respectively.
Fig. 3.2. *In vivo* quantification of VU730 to secretion of gel saliva from *A. gossypii* salivary glands. A) Concentration-dependent reduction of salivary sheath lengths after exposure to increasing concentrations of VU730 compared to control (gray bar). Bars represent average (n=30 individuals) sheath length and error bars represent SEM. Bars not labeled by the same letter represent statistical significance at \( P<0.05 \). B-C) Representative images of salivary sheaths from *A. gossypii* adults feeding on control (B) or VU730-treated sucrose solutions. Scale bars represent 10 µm.

3.3.2. Foliar applications of VU730 reduced xylem and phloem feeding by *A. gossypii*

Due to the observed reduction of salivary sheath secretions when exposed to VU730, we measured the feeding efficiency of *A. gossypii* through EPG analyses after foliar application of VU730. Aphids feeding on untreated (control) plants displayed stereotypical feeding patterns with clear probing events followed by C waveforms, E1 waveforms (salivation into phloem sieve-elements), E2 waveforms (phloem ingestion), and G waveforms (intake of xylem elements). Foliar application of VU730 did not reduce the proportion of aphids that probed on cotton leaves (Suppl. Fig. 3.1.A.), but aphids probing on VU730 treated plants reached xylem (G waveforms) 42.5% less than control aphids (Fig. 3.3.A.). Surprisingly, the mean time spent in xylem was not significantly different among control and aphids feeding on foliar treatments of VU730 (Fig. 3.3.B.). Further, aphids that successfully salivated (E1) and ingested (E2) phloem elements was significantly reduced to 8.3 ± 5.4% in VU730 treated plants compared to 70.8 ± 6.87% in control aphids (Fig. 3.3.C.). Although 8% (2 of 23) of aphids probing on plants treated with VU730 salivated and fed on phloem (Fig. 3.3.C.), these few individuals spent 5.6-fold less time in E1 (phloem salivation) and E2 phase (phloem ingestion) when compared to the control (Fig. 3.3.D.), which is a significant (\( P<0.01 \)) reduction.

3.3.3. Foliar applications of VU730 altered probing behavior of *A. gossypii*

The results above indicate that the Kir modulator VU730 inhibited salivary gland function and reduced aphid phloem feeding efficacy, but understanding how these inhibitors alter the duration of different aphid feeding behaviors can impact aphid survivorship and
horizontal transmission of plant pathogens. The time to first probe, which is defined as the time from the start of the EPG recording to the first intracellular puncture, was increased from 6.2 ± 1.4 minutes of control aphids to 18.7 ± 9.7 minutes in aphids exposed to VU730, indicating impedance of feeding initiation (Fig. 3.3.E.). In addition to the delayed probe initiation, the total number of probes and total probe duration over the entire feeding period were significantly ($P<0.05$) reduced by 1.6- and 1.7-fold, respectively, when compared to control aphids (Fig. 3.3.F. and 3.3.G.). Surprisingly, the duration of each probe that resulted in a potential drop was not significantly different among control and aphids feeding on foliar treatments of VU730 (Fig. 3.3.H.).
Fig. 3.3. Assessment of antifeedant capabilities of VU730 foliar treatment. Feeding behaviors as determined by EPG analysis over 4 hours for apterous cotton aphids infested on adaxial surface of a cotton leaf. Bars represent mean percentage of feeding behaviors and error bars represent SE. ‘*’ marks a significant difference through analysis by Mann-Whitney U Test comparing the Untreated Control (UTC) to VU730 1mM foliar application.

3.3.4. Toxicity of A. gossypii after foliar applications of VU730

No statistically significant difference in toxicity was observed after foliar application of 2 μg/cm² VU730 at 4- and 24- hours post infestation (Table 3.1.). Although not significantly
different when compared to control, a 2.5-fold increase in mortality was observed at 24 hours post exposure when compared to control that had 0 ± 0% mortality. No obvious signs of intoxication were observed. A 9.7-, 10.5-, 8.3-fold increase over control mortality were observed in VU730-treated leaves at 48-, 72-, and 96- hours post infestation, respectively (Table 3.1.).

3.3.5. Generation of Kir modulator water soluble concentrate (KM-WSC)

VU041 and VU730 have relatively high partition coefficients (cLogP: 3-4.5) that result in low water solubility and precipitate by themselves in water (Fig. 3.4.A-B, left vial in each panel) which prevents dispersal and translocation of VU041/VU730 throughout the plant tissues. The intrinsic solubility of VU041 and VU730 is less than 10 µg/mL in water and is 2 mg/mL in DMSO. In the presence of the natural solubilizer rubusoside, VU041 and VU730 were solubilized to 5 mg/mL in water and both KI-WSC were clear and transparent (Fig. 3.4. A-B, right vial in each panel). Compared with its intrinsic solubility, the water solubility of VU041 and VU730 were enhanced by more than 500-fold and was enhanced >2.5-fold when compared to DMSO. The VU041-WSC and VU730-WSC were freely dilutable in any concentrations that enabled them to remain soluble as they are translocated and diffused throughout the plant tissues. VU041-WSC remained stable in its appearances of being clear and transparent for 48 hours, but haziness was observed at time points greater than 48 hours post-solubilization and slight was observed 72 hours after solubilizing VU041-WSC in water. Vortexing the solution restored the dispersion for 2 hours before the haziness and precipitation reappeared. When it was clear and transparent, the water-soluble VU041 was in monodispersed nano-micelles with an average diameter of 3.1 ± 0.7 nm ranging from 2.0 to 4.5 nm (Fig. 3.4.C.). When VU041 water solution became cloudy and suspended after 3 days of being clear, VU041 was in bigger average particles of 4.2 ± 1.1 µm (Fig. 3.4.D.). Similar
to VU041, when VU730-WSC was clear and transparent with monodispersed nano-micelles had an average diameter of $3.6 \pm 0.5$ nm ranging from 2.5 to 5.5 nm (Fig 3.4.E.). Contrary to VU041, VU730 nano-micelles remained clear throughout the 7-day observation period.

Fig. 3.4. Enhancement of water solubility and particle size of VU041-WSC and VU730-WSC nano-micelles. 5 mg of VU041 (A) or VU730 (B) solubility in water (left) and after solubilization into WSC (right vial). C) clear VU041 nano-micelles in water showing an average diameter of $3.1 \pm 0.7$ nm with a range after 2 days in water. D) Suspended VU041 nano-micelles in water showing an average diameter of $4200 \pm 1700$ nm with a range from 3500 nm to 5500 µm after 3 days when the original clarity was lost. E) clear VU730 nano-micelles in water showing an average diameter of $3.6 \pm 0.5$ nm in diameter.
3.3.6. VU041-WSC and VU730-WSC was translocated to upper leaves and reduced phloem feeding.

EPG analyses were used to test if KI-WSC enables systemic movement of VU041 and VU730 throughout the plant tissue (Fig. 3.1.C.) by examining if feeding behavior on plants treated with KI-WSC mirrored phenotypes observed after foliar treatments of VU041 or VU730. Of the aphids that successfully probed on cotton, a mean of 87.04 ± 6.68% salivated into plant sieve-elements (E1 waveforms) in control groups. Importantly, aphids that fed on plants treated with WSC-only (no Kir inhibitor included, negative control) did not alter the proportion of aphids that probed the plant or performed G, E1, and E2 waveforms (Suppl. Fig. 3.2.A.-3.2.D.). VU730-WSC, but not VU041-WSC, reduced the percentage of aphids able to reach xylem from 95.00 ± 5.00% in control groups to 33.3 ± 14.4%, which is a statistically significant (P<0.05) reduction (Fig. 3.5.A.). To further assess the ability to feed on vascular bundles, we measured the time spent in xylem feeding. VU730-WSC significantly (P<0.05) reduced the time spent in xylem by 1.9-fold, but no change in time spent in xylem elements was observed with VU041-WSC (Fig. 3.5.B.). The lack of VU041-WSC influence to xylem feeding is correlated to the lack of change to xylem feeding after foliar applications of VU041.

Both VU041-WSC and VU730-WSC reduced the percentage of aphids that salivated in phloem sieve-elements (E1) by 1.8- and 3.5-fold when compared to the control treatments, respectively, which were statistically (P<0.05) significant reductions (Fig. 3.5.C.). Furthermore, the percentage of aphids that ingested phloem (E2) was significantly (P<0.01) reduced by 2.0- and 3.5-fold for VU041-WSC and VU730-WSC, respectively when compared to the control (Fig. 3.5.D.). Further, aphids infested on VU730-WSC treated plants led to a 1.5-fold increase in the time required for aphids to reach phloem after first initiating a probe when compared to the control, but this difference was not significant when compared to the control and VU041-WSC (Fig. 3.5.E.).
3.3.7. VU041-WSC and VU730-WSC treatments altered probing behavior of *A. gossypii*

In addition to KI-WSC effects to feeding on plant sieve elements, we aimed to quantify changes in specific feeding behavior of aphids pertinent to horizontal transmission of viruses. The proportion of aphids that initiated probes for VU041-WSC or VU730-WSC was not significantly (*P*<0.05) different among control (Suppl. Fig. 3.2.A.). However, both VU041-WSC and VU730-WSC increased the time from landing to first probe from 7.5 ± 1.8 minutes in control plants to 20.2 ± 6 minutes and 37.5 ± 14.9 minutes, respectively, which are statistically significant (*P*<0.01) increases when compared to control (Fig. 3.5.F.). Along with the delayed initiation of probing, the total probe duration was significantly (*P*<0.05) reduced by 1.4- and 2.3-fold in plants treated with VU041-WSC and VU730-WSC when compared to control, respectively (Fig. 3.5.G.). The average duration of each probe where aphid mouthparts penetrated the leaf epidermis was decreased by 4.1-fold in VU730-WSC, but not VU041-WSC (Fig. 3.5.H.).
Fig. 3.5. Assessment of translocalized movement of KI-WSC. Feeding behaviors as determined by EPG analysis over 7 hours for apterous cotton aphids infested on adaxial surface of a cotton leaf. Bars represent mean percentage of feeding behaviors and error bars represent SE. Bars not labeled by the same letter represent statistical significance as determined by one-way ANOVA followed by Tukey’s multiple comparisons test, or Welch’s ANOVA (due to unequal SD’s) with Dunnett’s T3 multiple comparison test.

3.3.8. VU041-WSC and VU730-WSC treated plants resulted in aphid mortality

EPG data indicate VU041 and VU730 are translocated to upper leaves after KM-WSC treatment of plant tissue to result in reduced phloem feeding (Figs. 3.5.C. and 3.5.D.), which
led us to hypothesize that aphids infested on upper leaves would have reduced survivorship due to lack of nutrient intake. Percent mortality from VU041-WSC or VU730-WSC treated plants was not significantly different than control for 4, 24 and 48 hr post-infestation (Table 3.1). However, mortality was increased by 1.6-, and 2-fold at 72 hours post infestation for VU041-WSC and VU730-WSC when compared to control groups (Table 1), respectively. Furthermore, we observed that percent mortality at 96 hours post exposure for VU041-WSC and VU730-WSC increased by 1.8-, and 2.1-fold, respectively when compared to the untreated control (Table 3.1).

Table 3.1. Toxicity assessments of VU730 foliar application and VU041 and VU730 dissolved in solubilizer to A. gossypii

Table 3.1. Toxicity of VU730 foliar application and VU041-WSC and VU730-WSC to adult apterous A. gossypii

<table>
<thead>
<tr>
<th>Foliar application, 2ug cm² (% mortality ± SE)</th>
<th>4 hours</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
<th>96 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>2.50 ± 2.50</td>
<td>5.00 ± 3.27</td>
<td>7.55 ± 3.66</td>
</tr>
<tr>
<td>VU730</td>
<td>0.00 ± 0.00</td>
<td>24.17 ± 12.84</td>
<td>41.25 ± 10.08*</td>
<td>62.50 ± 6.20***</td>
<td>72.08 ± 7.21***</td>
</tr>
</tbody>
</table>

*Translocation of VU041-WSC and VU730-WSC (% mortality ± SE)

<table>
<thead>
<tr>
<th>4 hours</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
<th>96 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.00 ± 0.00 A</td>
<td>0.00 ± 0.00 A</td>
<td>10.00 ± 3.33 A</td>
<td>21.43 ± 2.61 A</td>
</tr>
<tr>
<td>VU041-WSC</td>
<td>0.00 ± 0.00 A</td>
<td>4.00 ± 2.21 A</td>
<td>14.00 ± 4.00 A</td>
<td>34.00 ± 4.52 B</td>
</tr>
<tr>
<td>VU730-WSC</td>
<td>0.00 ± 0.00 A</td>
<td>3.91 ± 2.19 A</td>
<td>14.00 ± 2.21 A</td>
<td>42.36 ± 6.06 B</td>
</tr>
</tbody>
</table>

Asterisks represents statistical significance with * P<0.05, ***P<0.001 as determined by Mann-Whitney U Test compared to vehicle control

*Means labelled by different upper-case letters represents statistical significance (P<0.05) as determined by one-way ANOVA followed by Dunn’s Multiple comparisons test.
3.3.9. Detection and quantification of KM-WSC in the Leaf tissue of EPG recordings

We performed chemical analysis to identify VU041 and VU730 in the upper leaves of plants treated with KI-WSC to support data generated by leaf-core toxicity bioassays and EPG recordings. Retention times for VU730 and VU041 was determined to be 12.40 min and 17.66 min, respectively through Extracted Ion Chromatogram (EIC) analysis (Suppl. Fig. 3.3.A.). The exact mass for M+H was determined to be of 298.156 m/z and 364.163 m/z for VU730 and VU041, respectively. The KI-WSC’s were found to have a limit of Detection (LOD) of 3 ppb for VU041 and 1 ppb for VU730. The limit of quantification (LOQ) appeared to be 10 ppb for VU730 and VU041 when attempting to calculate quantities of compounds in leaf material. It was possible to detect <10 ppb of VU730 in 100% of the upper leaves of the VU730-WSC treated plants and VU041 was detected in 83.33% of upper leaves of VU041-WSC treated plants (Suppl. Fig. 3.3.B.). These exact masses at the same retention period were not detected in the WSC-only treated control (Suppl. Fig. 3.3.C.). An exact mass of 364.224 m/z was detected in both of WSC-only controls, which is similar but unequal to the m/z of VU041. We believe this peak to be attributed to plant material or other background noise due to its presence in all controls.

3.3.10. KM-WSC enabled translaminar transport of VU041 and VU730

Translocalization of VU041-WSC and VU730-WSC from lower to upper leaves led us to test the translaminar activity of VU041-WSC and VU730-WSC through EPG recordings on the underside of KI-WSC treated leaves (Fig. 3.1.B.). Treatment of Kir modulators with or without WSC did not significantly affect the percentage of aphids that initiated a probe on the abaxial surface of the leaf tissue (Fig. 3.6.A.). Aphids infested on plants treated with VU730-WSC reached xylem at a rate of 33.33 ± 8.33%, which was significantly (P<0.01) reduced from aphid feeding on control (95.0 ± 5.0%) and VU730 (no WSC) foliar-treated plants (83.33
± 6.03%; Fig. 3.6.B.). Aphids infested on plants treated with VU730-WSC salivated in sieve elements (E1) and ingested phloem (E2) at a rate of 41.67 ± 8.33%, which was significantly ($P<0.05$) reduced from aphid phloem feeding on control (91.67 ± 5.46%) and VU730 (no WSC) foliar-treated plants (95.24 ± 4.76%), which can be observed in Fig. 3.6.C. and 3.6.D. Aphids infested on plants treated with VU041-WSC salivated in sieve elements (E1) at a rate of 33.33 ± 13.09%, and passively ingested phloem (E2) at a rate of 27.78 ± 14.22% (Figs. 3.6.C. and 3.6.D). These data were significantly ($P<0.001$) reduced from aphid phloem feeding on control (91.67 ± 5.46%) and VU041 (no WSC) foliar-treated plants (72.92 ± 19.29%), which can observed in Fig. 3.6.C. and 3.6.D. Taken together, data indicate VU041-WSC and VU730-WSC enable trans-laminar movement to reduce the aphid’s ability to phloem feed, which was not observed after foliar application of Kir inhibitors without WSC (Figs. 3.6.C. and 3.6.D.).
Fig. 3.6. Assessment of translaminar movement of foliar treatment or WSC treatment with Kir inhibitors. Feeding behaviors as determined by EPG analysis over 7 hours for apterous cotton aphids infested on abaxial surface of a cotton leaf. Bars represent mean percentage of feeding behaviors and error bars represent SE. Bars not labeled by the same letter represent statistical significance as determined by one-way ANOVA followed by Tukey’s multiple comparisons test.

### 3.4. Discussion

Effective control of aphids is complexed by the global distribution of aphicide resistance to commercialized products and reduced insecticide registrations/approvals due to environmental concerns. The combination of increased aphid population growth and challenges for reducing vector populations has stimulated significant efforts to identify novel
modes of action and chemical scaffolds capable of inducing mortality while simultaneously altering plant feeding biology. Kir channels belong to a large super family of potassium channels and our understanding of insect Kir channels is emerging through the development of genetic and pharmacological tools. The function of Kir channels has been shown to be critical for proper function of multiple insect tissue systems and in particular, genetic depletion of pharmacological inhibition of Kir channels reduces the secretory activity of insect salivary glands and reduces sap- and blood feeding. Furthermore, the important physiological role Kir channels have to saliva secretion suggests they may be a valuable biochemical target for developing novel antifeedant insecticides.

The pyridine carboxamide molecule flonicamid has been registered for hemipteran control (BELEAF® 50SG) and reduces aphid salivation and sap feeding to induce lethality through starvation. Interestingly, recent evidence has suggested that flonicamid reduces feeding in the brown planthopper through inhibition of *Nilaparvata lugens* Kir1 channels and similarly, we have previously shown that foliar application of the Kir channel inhibitor, VU041, reduces aphid salivation and feeding on sieve elements to result in toxicity. We expanded previous datasets through assessment of biological feeding parameters of VU730, which is a closely related structural analog to VU041, because it is a more potent inhibitor of Kir1 through thallium-flux assays and has cleaner ancillary pharmacology. Significant reduction of gel-saliva secretions in VU730-treated aphids was correlated to near elimination of salivation and ingestion of phloem sieve elements (Fig. 3.2.), which was similar to our previous reports with VU041. Thus, the removal of the trifluoro group from the pyrazole and replacement of the cyclohexane in VU041 with a tetrahydropyran group to generate VU730 did not reduce the inhibitory activity to salivary gland function or antifeedant activity (Fig. 3.3.C. and 3.3.D.). In addition to reduced phloem feeding, aphids infested on VU730 treated leaves or VU730-WSC treated plants had a reduced ability to feed on xylem sieve
elements, which was not previously documented with VU041 \textsuperscript{109} or VU041-WSC (Fig. 3.3.A.). The reduced xylem feeding is potentially due to increased hydrophilicity of VU730 compared to VU041 that was produced by the removal of the trifluororo group combined with addition of a tetrahydropyran groups that reduced cLogP value of VU730 by 1.5 units. Xylem consumption is highly relevant to aphid survivorship because ingestion of xylem sap maintains their water balance and prevents dehydration \textsuperscript{121}. No acute toxicity was reported with VU041 or flonicamid due to their ability to reach xylem but not phloem that led to starvation at 72+ hours post infestation \textsuperscript{89,109}, yet VU730 reduced xylem feeding after foliar treatment of plants (Fig. 3.3.A.) suggesting mortality observed at 24 hours post infestation (Table 3.1.) is due to dehydration stemming from reduced xylem feeding (Fig. 3.3.A. and 3.3.B.). The observed increase in VU730 mortality at 72+ hours mirror the time-course mortality of VU041 and flonicamid that we speculate is due to starvation stemming from reduced phloem feeding.

Aphid mediated transmission of plant viruses by aphids occurs during distinct feeding events, such as probing for non-persistent viruses or phloem feeding for persistent viruses \textsuperscript{19,35}. We have shown that initiation of aphid probing, and thus transmission of non-persistent viruses, occur between 4-6 minutes after landing on the plant \textsuperscript{122} that indicates it is necessary to prevent plant probing or induce mortality within 4-6 minutes after aphid landing. While no change in time to first probe or probe duration was observed with VU041 \textsuperscript{109}, the time to first probe for aphids infested on leaves treated with VU730 was increased to approximately 15 minutes, which is 3-fold greater than control aphids (Fig. 3.3.E.). Further, the time spent in the probe was significantly reduced that may suggest a reduced probability of reaching the transmission phase of feeding where pathogens are horizontally transmitted from the aphid to the plant. Considering the near elimination of phloem feeding, the dramatic increase in time to first probe, and the reduced time spent in the probe provides strong indication that VU730
will interfere with horizontal transmission of aphid-mediated non-persistent and persistent viruses. Of course, downstream work is needed to verify this speculation.

Unlike flonicamid, which is a polar molecule with a LogP value 0.8, the Kir inhibitors VU041 and VU730 are non-polar and do not have inherent plant systemic movement that restricts the use of VU041 and VU730 to foliar applications and contact exposure of aphids. Foliar applications of aphicides to plants has been the common method of treatment, yet the recent concerns for environmental contamination and chemical exposure of pollinator and beneficial insects has led to steep criticism of foliar sprays\textsuperscript{123,124}. Although similar concerns have been documented with systemic neonicotinoids\textsuperscript{123}, concern is mainly focused on the persistence of this class of insecticides and not necessarily the method of delivery. Poor aqueous solubility is a common obstacle for the systemic movement of agrochemicals that is mirrored by the pharmaceutical industry where 87% of pharmaceuticals have a water solubility of >65 µg/mL while the minimum acceptable solubility is 52 µg/mL\textsuperscript{118}. Thus, the pharmaceutical industry has employed complexing agents (\textit{e.g.} cyclodextrins), cosolvents (\textit{e.g.} ethanol), surfactants (\textit{e.g.} Tween), emulsifiers (\textit{e.g.} glycerol), or nanosuspension techniques to increase the water solubility and these technologies have been also tested for agrochemicals\textsuperscript{125-129}. Complexing agents and some nanosuspension techniques increase solubility through formation of non-covalent stoichiometric association to form inclusion complexes with the insoluble drug\textsuperscript{130,131}. However, these approaches are often plagued with rapid precipitation of the active ingredient and loss of water solubility that reduces utility of these technologies for agrochemicals. Rubusoside, a naturally occurring terpene glycoside present in Chinese sweet leaf tea leaves (\textit{Rubus suavissimus})\textsuperscript{119}, has been shown to enhance the water solubility of multiple structurally distinct drugs by 5- to 1000-fold that is stable for multiple days after solubilization\textsuperscript{116,132}. Rubusoside is non-toxic and is listed on the Generally Recognized as Safe (GRAS) list that, when combined with the enhancement of solubility of pharmaceuticals,
provided the impetus to test the ability of rubusoside to enhance water solubility of lipophilic VU041 and VU730. Indeed, water solubility of VU041 and VU730 was increased by approximately 500-fold after formulation with rubusoside (Fig. 3.4.) and importantly, the biological activity of VU041 and VU730 was retained after translocalization and translaminar movement throughout the plant (Fig. 3.5.-3.6.). Although we were able to detect the presence of VU041 and VU730 in upper leaves of KI-WSC treated plants, it was not possible to quantify the concentration of chemicals due to the presence of compounds within the leaf tissue at a similar retention time. Further optimization of tissue processing to remove plant-derived compounds is likely to enable quantification of the compounds. Although not ideal, we are confident VU041 and VU730 reached the upper leaves at a relatively high concentration due to the retainment of biological activity where a >75% inhibition of phloem feeding was observed with VU730-WSC (Figs. 3.5.C. and 3.5.D.).

The data presented in this study further support the physiological role and toxicological relevance of aphid Kir channels by providing evidence these channels are critical to the role of aphid salivary gland function and phloem feeding. These data indicate VU730, like VU041, add to the few commercialized aphicides that reduce vector abundance while simultaneously altering feeding behaviors that are relevant to horizontal transmission of plant viruses. Additionally, the enhancement of water solubility and plant systemicity while retaining biological activity of VU041 and VU730 with rubusoside provides a novel approach for increasing options for delivery of lipophilic insecticides to control aphid pests in agricultural systems. Solubility enhancement through the formation of water-soluble nanomicelles enabled better absorption of Kir inhibitors into the applied leaf area to generate systemic movement through the plant that was not possible with foliar application of non-WSC solubilized Kir inhibitors. Further determination of the effect of particle size (e.g., nanomicelles vs micro-micelles) on bioavailability may be meaningful in finalizing formulations of
Kir inhibitors or other agrochemicals. Downstream studies should be directed to testing the anti-transmission properties of Kir channel inhibitors as well as the potential of other diterpene glycosides to increase water solubility and plant systemicity for other lipophilic aphicides.

3.5. Supplemental figures

Supplemental Figure 1: Mean percentage of apterous cotton aphids that initiated a probe on cotton after VU730 foliar treatment. Feeding behaviors as determined by EPG analysis over 7 hours for apterous cotton aphids infested on abaxial surface of a cotton leaf. Bars represent mean percentage of feeding behaviors and error bars represent SE. ‘**’ marks a significant difference through analysis by Mann-Whitney U Test comparing the Untreated Control (UTC) to VU730 1mM foliar application.
Supplemental Figure 2: Assessment of WSC-only on aphid feeding behavior. Feeding behaviors as determined by EPG analysis over 7 hours for apterous cotton aphids infested on adaxial surface of a cotton leaf. Bars represent mean percentage of feeding behaviors and error bars represent SE. For A) ‘Letters’ marks a significant difference through analysis by One-way analysis of variance and a Tukey’s multiple comparisons test. For B) - C) Asterisk marks a significance significant difference through analysis by Mann-Whitney U Test comparing the Untreated Control (UTC) to WSC-only.
Supplemental Figure 3: Chromatogram plots for the detection of VU730 and VU041. A) Detection of exact masses of VU730 and VU041 that had been spiked at 1000PPB in processed leaf material. B) Detection of exact masses of VU730 and VU041 at retention times of 12.40 minutes and 17.66 minutes, respectively. C) No detection of exact masses of VU730 and VU041 at retention times of 12.40 minutes and 17.66 minutes, respectively. Black arrows represent anticipated peak for VU041 and VU730.

3.6. Acknowledgements

We thank Mr. Mark (Jeff) Murray for his assistance with rearing of aphids and cotton plants used for this work. This research was partly supported by the United States Department of Agriculture-Agriculture and Food Initiative award (2020-67013-31858, PI: Swale). The USDA is an equal opportunity provider and employer.
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Vita

Flinn O’Hara was born in London and raised on the South coast of the United Kingdom. He attended The University of Manchester from 2014 to 2018 where he graduated with a bachelor’s degree in Developmental Biology (honors) with Industrial or Professional Experience. He spent 2 years working as a Science Graduate for Rentokil Pest Control before joining the Swale Lab in the Department of Entomology in 2020 where he plans to earn his master’s degree in August of 2022. The thesis project he has finished is to create a baseline understanding of how commercial aphicides affect survivorship and feeding behavior to the cotton aphid and compare these results to inward rectifier potassium channel inhibitors as a novel antifeedant aphicide. He plans to remain in the Swale Lab at the University of Florida, Gainesville to pursue his Ph.D. degree in Entomology.